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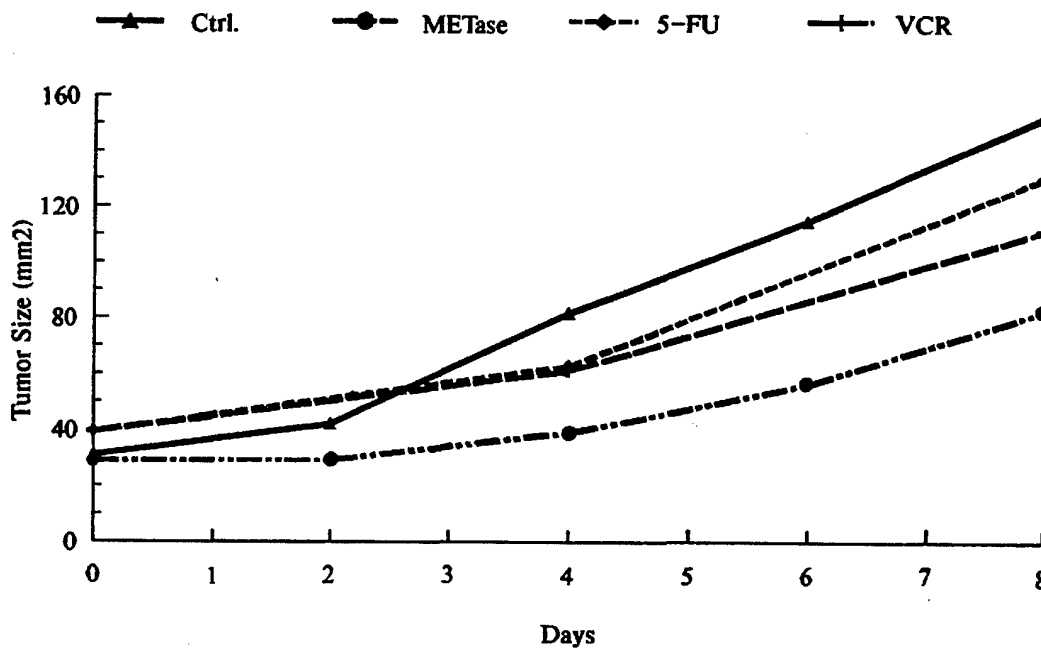
(57) Abstract

The invention provides highly pure, endotoxin free methioninase compositions that have been prepared from natural material and from a recombinant host. The invention further provides methioninase that has been coupled to a polymer such as polyethylene glycol. The isolation of a methioninase encoding nucleic acid molecule and the generation of high expression vectors containing the methioninase encoding nucleic acid molecule is also provided.

USE OF METHIONINASE IN ANTI-METHIONINE AND ANTI-HOMOCYSTEINE
CHEMOTHERAPY

Technical Field

5   The present invention relates to methioninase compositions,
methods of purification of methioninase, methods of coupling
methioninase to a polymer such as PEG, methods using
methioninase in antimethionine and anti-homocysteine
chemotherapy. More particularly, the methods of the invention
10   include the use of methioninase in cancer therapy,
cardiovascular therapy, and tumor imaging and diagnosis. The
present invention further relates to recombinant DNA technology.
The present invention particularly provides expression modules
that encode methioninase.

15

Background
       Therapeutic drug-based treatment of cancer is directed at
the use of medicinals which selectively inhibit or kill the
cancer cells while not harming normal tissue function beyond
20   acceptable amounts. The difficulty with conventional
chemotherapy has been the toxicity of therapeutic drugs for
normal tissue.
       Many tumors have been shown to have absolute requirement
for methionine in a variety of cell types and evaluated tumor
25   tissues, including tumors of the colon, breast prostate, ovary,
kidney, larynx melanoma, sarcoma, lung, brain, stomach and
bladder as well as leukemias and lymphomas. Methionine
dependence has been defined as an inability of tumors to grow
when methionine is replaced by homocysteine in the growth
30   medium. See, for example, Chello et al., Cancer Res., 33:1898-
1904, 1973; and Hoffman, Anticancer Res., 5:1-30, 1985.
       Methionine depletion has been shown to selectively
synchronize methionine-dependent tumor cells into late S/G$_2$ phase
of the cell cycle. Hoffman et al, Proc. Natl. Acad. Sci. USA,
35   77:7306-7310, 1980. Using the combination of methionine

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deprivation, followed by repletion of methionine coupled with
exposure to an antimitotic agent, termed antimethionine
chemotherapy, tumor cells have been selectively eliminated from
cocultures of normal and tumor cells, resulting in cultures of
5   normal cells proliferating vigorously. Stern *et al.*, J. Natl.
Cancer Inst., 76:629-639, 1986.

However, in order for methionine-dependent chemotherapy to
be conducted *in vivo*, it is necessary to have a means to
effectively deplete serum of circulating methionine. Methionine
10   depletion methods have not been described that reduce
circulating methionine levels *in vivo* in a manner sufficient to
be effective in antitumor therapies.

Methioninase, an enzyme which degrades methionine, has been
purified from a variety of bacterial sources, and has been
15   reported to slow the rate of tumor cell proliferation *in vitro*.
Kreis *et al.*, Cancer Res., 33:1862-1865, and 1866-1869, 1973;
Tanaka *et al.*, FEBS Letters, 66:307-311 1976; Ito *et al.*, J.
Biochem. 79:1263-1272, 1976; and Nakayama *et al.*, Agric. Biol.
Chem. 48:2367-2369, 1984.

20   Kreis *et al.*, Cancer Res. 33:1866-1869, 1973, have
described the use of highly impure methioninase preparations
isolated from *Clostridium sporgenes* at 1150 units/kg/day to
inhibit growth of carcinosarcoma cells implanted in a mouse
model. Although the enzyme apparently reduced primary tumor
25   cell growth, it was not reported to reduce the T/C (treated
versus control) ratio of tumor diameter below 50%, and was not
reported to have any effect on metastasis. The authors also
indicated that tumor specificity of the methioninase cannot be
expected without other unspecified interventions, and further do
30   not comment on the possibly that endotoxin, or other components
of the impure preparation, were responsible for the effects
observed. The only toxicity studies reported were absence of
animal body weight loss after the duration of the treatment, and
negative gross examination for toxicity. Further, the authors
35   report that the enzyme had a serum half life of 4 hours. The

enzyme preparation was only semi-pure and contained significant amounts of endotoxin complicating the interpretation of the results.

5   Further, Kreis was unable to reduce serum methionine levels to below about 8% of control. his is likely attributable to the high K_m (approx. 90mM) of the *Clostridium* enzyme. The methioninase preparation was reported to be unstable and only a 2% recovery (yield) could be achieved in its purification.

       Kreis *et al.*, Cancer Res. 33:1866-1869, 1973, further
10   reported the use of a methionine-free diet as a means to deplete methionine as an antitumor therapy. However, the authors reported that the diet did not slow tumor growth as effectively as the use of an impure preparation of methioninase and resulted in the undesirable side effect of continuous loss of weight of
15   the animal. The authors did not report the use of methionine deficient diets combined with methioninase treatment, and did not study cell synchronization.

       The priority applications of the present invention disclose effective chemotherapy of tumors directed at effectively
20   reducing the amount of methionine as to provide a beneficial antitumor effect without deleterious injury using methioninase. The present invention improves the disclosed therapeutic and diagnostic methods and composition by providing a source for producing commercially viable quantities of highly pure
25   recombinant methioninase.

Brief Description of the Invention

       It has now been discovered that methioninase, either in PEGylated form or as produced and purified as a highly pure,
30   endotoxin free recombinant form, can deplete levels of methionine in mammals without harm, can be effectively used to selectively inhibit tumor growth and further can be used to selectively arrest and thereby synchronize tumor cells for antimitotic chemotherapy.

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It has also been discovered that methionine depletion is most effective when several different methods are used synergistically to substantially reduce effective methionine levels. These methods include methionine starvation using a

5 methionine-free diet, competitive inhibition of methionine by use of inhibitors that compete with methionine for methionine-utilizing enzymes, chemotherapeutic agents that take advantage of the specific tumor selective cell-cycle block induced by methionine depletion and combinations thereof.

10 Thus, the invention describes a method for inhibiting tumor cell growth comprising contacting a population of tumor cells with a therapeutically effective amount of methioninase as herein defined for a time period sufficient to induce cell cycle stasis in cells of the population, and form static tumor cells.

15 By "contacting" is meant that the therapeutic compositions of the present invention are placed in close enough proximity to the targeted tumor such that a tumor-inhibiting effect is observed, or that the therapeutic compositions are placed in a medium, such as a nutrient medium or blood, such that the level

20 of methionine in the medium is decreased to a level where the growth of tumor cells in the medium are inhibited. By "static" tumor cells is meant tumor cells that are growth inhibited. The method can be practiced _in vitro_ or _in vivo_. The contacting of the tumor cells does not need to be direct and may be executed

25 by contacting the nutrient medium containing the tumor cells, either _in vitro_ or _in vivo_. The contacting may also be accomplished by contacting circulating blood with methioninase. Such circulating blood may or may not contain tumor cells as the tumors may be solid tumors that do not circulate. By

30 "inhibiting tumor cell growth" is meant that administration of the compound of the invention reduces the growth of a tumor, as measured by, for example, size, by 10% to 100%, more preferably by 34% to 79%, and more preferably by 55% to 68% when compared to a tumor that has not been treated with the compound.

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Methioninase is used as an antitumor reagent to slow or stop cell division by depleting methionine. This effect can be enhanced with competitive inhibitors of methionine. In addition, methioninase can be used in combination with anti-

5   mitotic and other cell-cycle specific cytotoxic agents to increase the therapeutic effectiveness of the antimitotic or other cell-cycle specific cytotoxic agents by inducing cell cycle synchronization.

Methioninase is also used in homocysteine depletion

10   chemotherapy to reduce the risk of and to treat cardiovascular disease. Methioninase is also used to deplete blood and tumor [^{12}C] methionine prior to repletion with [^{11}C] methionine to detect and diagnose tumors in the body at ultra-high resolution.

In one embodiment, the tumor cells are first subjected to a

15   methionine-starvation step to first reduce the level of methionine. Optionally, the methionine starvation may be accompanied by homocysteine to reduce toxicity to normal cells, thereby increasing the specificity of the therapy.

Another related aspect of the present invention features

20   the use of the present methionine-depletion methods to arrest tumor cells in late S/G$_2$ phase of the cell cycle, followed by treatment to synchronously initiate cell-cycling and the administration of a cell cycle-specific cytotoxic agent such as an antimitotic to selectively and effectively kill tumor cells.

25   Those of ordinary skill in the art are familiar with cell cycle-specific cytotoxic agents that are toxic to cells of particular phases of the cell cycle. The method comprises the steps of:

(a)   contacting the static tumor cells produced by the previous methionine-depletion step with a cell cycle-inducing

30   amount of methionine to initiate cell-cycling of the static tumor cells forming cycling cells, and

(b)   contacting the cycling cells with a cell cycle-specific cytotoxic agent in an amount sufficient to inhibit mitosis of the mitotic cells, thereby inhibiting tumor cell

35   growth.

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In one embodiment, the method comprises the steps of

(a) contacting the static tumor cells produced by the previous methionine-depletion step with a cell cycle-inducing amount of methionine to initiate mitosis of the static tumor
5   cells forming mitotic cells, and

(b) contacting the mitotic cells with an anti-mitotic agent in an amount sufficient to inhibit mitosis of the mitotic cells, thereby inhibiting tumor cell growth.

The invention also features the use of methioninase to
10   lower homocysteine levels in patients to reduce the risk of, and to treat, cardiovascular diseases.

The invention provides a method of depleting methionine by administration of methioninase for tumor diagnosis and imaging.

Also featured are therapeutic compositions for methionine
15   depletion therapy comprising a therapeutically effective amount of substantially isolated methioninase having a specific activity of at least about 10 to about 50 units methioninase activity per milligram (mg) protein and less than about 1 to about 100 ng of endotoxin per mg protein. In the preferred
20   embodiment, the therapeutic composition contains less than 10 nanograms endotoxin per mg protein, together with a pharmaceutically acceptable carrier.

The therapeutic compositions of the present invention also include an endotoxin-free methioninase. In one embodiment, the
25   methioninase is prepared using a novel and efficient method from Pseudomonas putida grown under improved fermentation conditions. To this end, the present invention also provides a method of isolating an improved, high-methioninase-producing strain of P. putida. In another embodiment, the methioninase is produced
30   from a recombinant host containing an expression module that encodes methioninase.

The methioninase compositions of the present invention are also provided in a chemically modified form by coupling of the methioninase to polymers such as polyethylene glycol (PEG). The
35   PEG-modified methioninase of the present invention exhibits high

methionine depletion activity, an extended half-life, and low immunogenicity.

The methoninase compositions of the present invention are also provided as a recombinant protein produced using an

5   expression vector such as a high expression vectors are herein defined. Methoninase produced using a high expression system can be readily purified using the methods described herein to obtain a methoninase composition that is endotoxin free and highly pure, having a specific activity of about 10 to about 50

10   units/mg methoninase.

The invention further provides vectors containing nucleic acid molecules that express unexpectedly high levels of methoninase. Such vectors, for example those that utilize the T7 RNA polymerase promoter, have been used to produce

15   methoninase at about 1 to about 4 grams/liter with a specific activity of about 2.6 to about 5 in the crude homogenate, representing from about 10 to about 20% of the total cellular protein under appropriate incubation conditions.

The invention further provides gene therapy methods that

20   utilize a cloned methoninase gene that can be used in place of compositions containing methoninase for the herein disclosed methods. Cells can be altered to express methoninase within a patient as a means of supplying a therapeutic amount of methoninase to a patients serum.

25   Other features, advantages and related embodiments of the present invention will be apparent based on the disclosures contained herein.

Brief Description of the Drawings

30   Figure 1 is a compilation of 6 graphs illustrating the growth of tumor cell lines in methione-free, homocysteine-free, or methione and homocysteine-free media.

Figure 2 is a graph that illustrates the efficacy of methoninase for reducing the level of human lung tumor (H460)

growth in mouse xenografts. The results of 5-FU and vincristine
treatment are also illustrated.

    Figure 3 is a graph that illustrates the effect of
methioninase, 5-FU, and vincristine on the body weight of mice
implanted with a human lung tumor.(H460)

    Figure 4, 5 and 6 are graphs of the pharmacokinetics of
methioninase and methionine after administration of methioninase
to three human patients. The percentage of methioninase
activity is measured as a relative percentage of activity of the
starting preparation of methioninase. The percentage of
methionine is measured as a relative percentage of the
methionine concentration before administration of methioninase.

    Figure 7 is an illustration of the methioninase-
homocysteine metabolic cycle.

    Figure 8 provides the nucleotide sequence and corresponding
amino acid sequence of a methioninase encoding DNA molecule
isolated from *P. putida*.

    Figure 9 provides a diagram of representative high
expressionmodules contained in vectors.

    Figure 10 provides an outline of the purification steps
used to obtain highly pure, endotoxin free methioninase.

    Figure 11 provides an overview of typical purity and
recovery yields for rMETase using the purification method herein
described.

    Figure 12 provides an example of the purity of rMETase
produce by the present methods.

    Figure 13 provides an activity profile for different
rMETase formulations.

    Figure 14 provides HPLC analysis data for PEG-rMETase.

    Figure 15 provides HPLC analysis data for PEG-rMETase.

    Figure 16 provides the pharmacokinetics of PEG-rMETase in
mice.

    Figure 17 provides the growth inhibition provided by
rMETase on KB3-1 cells *in vitro*.

Figure 18 provides efficacy of rMETase against KB3-1 cells in nude mice.

Figure 19 provides the toxicity of rMETase on body weight in nude mice.

5   Figure 20 provides the toxicity of rMETase on blood cells in nude mice with KB3-1 cells.

Figure 21 provides the toxicity of rMETase in BALB/C mice.

Figure 22 provides the toxicity of rMETase in BALB/C mice.

Figure 23 provides a toxicity evaluation of methioninase in 10   human patients.

Figure 24 provides a pharmacokinetic evaluation of rMETase in a human patient.

Figure 25 provides data demonstrating growth inhibition of rMETase on H460 and HT29 in nude mice.

15   Figure 26 provides a comparison of sensitivities to rMETase between normal cells and human cancer cells *in vitro*.

The drawings are not necessarily to scale, and certain features of the invention may be exaggerated in scale and shown in schematic form in the interest of clarity and conciseness.

20

Description of the Invention

A.   Definitions

"Amino acid residue" refers to an amino acid formed upon chemical digestion (hydrolysis) of a polypeptide at its peptide 25   linkages. The amino acid residues described herein are preferably in the "L" isomeric form. However, residues in the "D" isomeric form can be substituted for any L-amino acid residue, as long as the desired functional property is retained by the polypeptide. NH$_2$ refers to the free amino group present 30   at the amino terminus of a polypeptide. COOH refers to the free carboxy group present at the carboxy terminus of a polypeptide. In keeping with standard polypeptide nomenclature (described in J. Biol. Chem. 243:3552-59, 1969, and adopted at 37 C.F.R. 1.822(b)(2)), hereby incorporated by reference.

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It should be noted that all amino acid residue sequences
represented herein by formulae have a left-to-right orientation
in the conventional direction of amino terminus to carboxy
terminus. In addition, the phrase "amino acid residue" is
5   broadly defined to include the amino acids listed in the Table
of Correspondence and modified and unusual amino acids, such as
those listed in 37 CFR 1.822(b)(4), and incorporated herein by
reference. Furthermore, it should be noted that a dash at the
beginning or end of an amino acid residue sequence indicates a
10   peptide bond to a further sequence of one or more amino acid
residues or a covalent bond to an amino-terminal group such as
NH$_2$ or acetyl or to a carboxy-terminal group such as COOH.

"Recombinant DNA (rDNA) molecule" refers to a DNA molecule
produced by operatively linking two DNA segments. Thus, a
15   recombinant DNA molecule is a hybrid DNA molecule comprising at
least two nucleotide sequences not normally found together in
nature. rDNA's not having a common biological origin, i.e.,
evolutionarily different, are said to be "heterologous".

"Vector" refers to a rDNA molecule capable of autonomous
20   replication in a cell and to which a DNA segment, e.g., gene or
polynucleotide, can be operatively linked so as to bring about
replication of the attached segment. Vectors capable of
directing the expression of genes encoding for one or more
polypeptides are referred to herein as "expression vectors".
25   Particularly important vectors allow convenient expression of a
methioninase protein of this invention.

B.   Methods For Using Methioninase as an Antitumor Agent
     I.   Methionine Depletion
30   Methioninase is used herein as an antitumor agent in a
variety of modalities for substantially depleting methionine
from a tumor cell, tumor tissue or the circulation of a mammal
with cancer, or for depletion of methionine where its depletion
is considered desirable, as described further herein.

Whereas prior methods can reduce the levels *in vivo* of
methionine to no lower than about 35 micromolar (uM), no methods
are known which can safely, easily and rapidly reduce methionine
levels substantially below about 10 uM. By substantially is

5   meant at least detectably below 10 uM, preferably less that 1
uM, more preferably less than 0.1 uM, and most preferably to
non-detectable levels, using conventional methionine detection
methods. Methionine can be measured in aqueous solutions,
including body fluids such as blood, plasma and serum, by a

10   variety of methods. An exemplary method is reverse-phase FPLC
using methionine standards.

Depletion can be conducted *in vivo*, in the circulation of a
mammal, *in vitro* in cases where methionine depletion in tissue
culture or other biological mediums is desired, and in ex vivo

15   procedures where biological fluids, cells or tissues are
manipulated outside the body and subsequently returned to the
body of the patient mammal.

Depletion of methionine from circulation, culture media,
biological fluids or cells is conducted to reduce the amount of

20   methionine accessible to the material being treated, and
therefore comprises contacting the material to be depleted with
a methionine-depleting amount of methioninase according to the
present invention under methionine-depleting conditions as to
degrade the ambient methionine in the material being contacted.

25   Because tumor cells are dependent upon their nutrient
medium for methionine, the depletion may be directed to the
nutrient source for the cells, and not necessarily the cells
themselves. Therefore, in an *in vivo* application of the present
invention, the methioninase can be contacted with the nutrient

30   medium for a population of tumor cells. In this embodiment, the
medium can be blood, lymphatic fluid, spinal fluid and the like
bodily fluid where methionine depletion is desired.

A methionine-depleting amount can vary widely depending
upon the application, and typically depends upon the amount of

35   methionine present in the material, the desired rate of

depletion, and the tolerance of the material for exposure to
methioninase. Methionine levels in a material, and therefore
rates of methionine depletion from the material, can readily be
monitored by a variety of chemical and biochemical methods well

5   known in the art. Exemplary methionine-depleting amounts are
described further herein, and can range from 0.001 to 100 units
(U) of methioninase, preferably about 0.01 to 10 U, and more
preferably about 0.1 to 5 U methioninase per milliliter (ml) of
material to be treated.

10   Methionine-depleting conditions are buffer and temperature
conditions compatible with the biological activity of the
methioninase enzyme, and include moderate temperature, salt and
pH conditions compatible with the enzyme. Exemplary conditions
include about 4-40 degrees Centigrade (°C), ionic strength

15   equivalent to about 0.05 to 0.2 M NaCl, and a pH of about 5 to
9, although physiological conditions are preferred.

         In a preferred embodiment, the invention contemplates
methods of using methioninase as an antitumor agent, and
therefore comprises contacting a population of tumor cells with

20   a therapeutically effective amount of methioninase for a time
period sufficient to induce cell cycle stasis in cells of the
population, and form static tumor cells.

         As described herein, the placement of tumor cells in cell
cycle stasis is desirable for a variety of reasons, including

25   but not limited to, selectively slowing tumor growth, killing
tumor cells by maintaining the cells in stasis for such
prolonged time periods as to produce cell death, and preparation
of a population of tumor cells for cell cycle synchronization
prior to a subsequent chemotherapeutic treatment, and the like.

30   The time period required to induce cell cycle stasis by
contact with methioninase depends on several factors, such as
the amount of methioninase contacted with the cells or the
medium containing the cells, the amount of methionine, specific
activity of the enzyme, temperature and other reaction

35   conditions affecting reaction rate, and the like parameters

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readily controllable by the practitioner. Typical time periods
are 10 minutes to about 30 days, preferably about 1 hour to 20
days, and more preferably about 1 to 10 days.

Cell cycle stasis is a condition in which the cell is not
5   dividing nor cycling through the full cycle of events, the
phases being individually referred to as G_0, G_1, G_2 and S phases.
Upon methionine depletion, it is believed that the cell stops
cycling in the late S/G_2 phase as evidenced by the accumulation
of DNA relative to normal resting cells. Methods for
10 identifying cell cycle stasis can be determined by a variety of
histological methods, and can be evaluated by cell culture, and
involve measuring DNA content of a population of cells as
described in the Examples.

Tumors for which the present treatment methods are useful
15 include any malignant cell type such as is found in a solid
tumor or a hematological tumor. Exemplary solid tumors can
include, but are not limited to a tumor of an organ selected
from the group consisting of pancreas, colon, cecum, stomach,
brain, head, neck, ovary, kidney, larynx, sarcoma, lung,
20 bladder, melanoma, prostate and breast. Exemplary hematological
tumors include tumors of the bone marrow, T or B cell
malignancies, leukemias, lymphomas, blastomas, myelomas, and the
like.

In one embodiment, the contacting *in vivo* is accomplished
25 by administering, by intravenous or intraperitoneal injection, a
therapeutically effective amount of a physiologically tolerable
composition containing methioninase of this invention to a
patient, thereby depleting the circulating methionine source of
the tumor cells present in the patient. The contacting of
30 methioninase can also be accomplished by administering the
methioninase into the tissue containing the tumor cells.

A therapeutically effective amount of a methioninase is a
predetermined amount calculated to achieve the desired effect,
i.e., to deplete methionine in the tumor tissue or in a

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patient's circulation, and thereby cause the tumor cells to stop
dividing.

     Thus, the dosage ranges for the administration of
methioninase of the invention are those large enough to produce

5   the desired effect in which the symptoms of tumor cell division
and cell cycling are reduced. The dosage should not be so large
as to cause adverse side effects, such as hyperviscosity
syndromes, pulmonary edema, congestive heart failure, and the
like. Generally, the dosage will vary with the age, condition,

10 sex and extent of the disease in the patient and can be
determined by one of skill in the art.

     The dosage can be adjusted by the individual physician in
the event of any complication.

     A therapeutically effective amount of a methioninase of

15 this invention is typically an amount such that when
administered in a physiologically tolerable composition is
sufficient to achieve a intravascular (plasma) or local
concentration of from about 0.001 to about 100 units (U) per ml,
preferably above about 0.1 U, and more preferably above 1 U

20 methioninase per ml. Typical dosages can be administered based
on body weight, and are in the range of about 5-1000 U/kilogram
(kg)/day, preferably about 5-100 U/kg/day, more preferably about
10-50 U/kg/day, and more preferably about 20-50 U/kg/day.

     In preferred methods, a methioninase used is substantially

25 free of endotoxin, as discussed further herein. Particularly
preferred is the use of recombinantly produced methioninase that
is substantially free of endotoxin.

     The methioninase can be administered parenterally by
injection or by gradual infusion over time. Methioninase can be

30 administered intravenously, intraarterially, intraperitoneally,
orally, intramuscularly, subcutaneously, intracavity,
transdermally, dermally, can be delivered by peristaltic means,
or can be injected directly into the tissue containing the tumor
cells or can be administered by a pump connected to a catheter

35 that may contain a potential biosensor or methionine.

The therapeutic compositions containing methioninase are
conventionally administered intravenously, as by injection of a
unit dose, for example. The term "unit dose" when used in
reference to a therapeutic composition of the present invention

5   refers to physically discrete units suitable as unitary dosage
for the subject, each unit containing a predetermined quantity
of active material calculated to produce the desired therapeutic
effect in association with the required diluent; i.e., carrier,
or vehicle.

10   The compositions are administered in a manner compatible
with the dosage formulation, and in a therapeutically effective
amount. The quantity to be administered depends on the subject
to be treated, capacity of the subject's system to utilize the
active ingredient, and degree of therapeutic effect desired.

15   Precise amounts of active ingredient required to be administered
depend on the judgment of the practitioner and are peculiar to
each individual. However, suitable dosage ranges for systemic
application are disclosed herein and depend on the route of
administration. Suitable regimes for initial administration and

20   booster shots are also contemplated and are typified by an
initial administration followed by repeated doses at one or more
hour intervals by a subsequent injection or other
administration. Exemplary multiple administrations are
described herein and are particularly preferred to maintain

25   continuously high serum and tissue levels of methioninase and
conversely low serum and tissue levels of methionine.
Alternatively, continuous intravenous infusion sufficient to
maintain concentrations in the blood in the ranges specified for
in vivo therapies are contemplated.

30

       II.   Methionine Depletion Methods
       In practicing the present methods, methionine depletion by
contacting a methionine-containing material with methioninase as
described earlier can be accompanied by one or more

35   supplementary methionine depletion steps. Furthermore, the

invention contemplates a variety of treatment procedures where
the depletion of methionine is desirable, as described further
herein.

5   a.   Methionine Starvation
        For example, a methionine starvation step may be used to
first reduce the ambient levels of methionine, wherein the
starvation step involves contacting the tumor cell(s) with
methionine-free nutrients for a methionine starvation time
10   period that is conducted prior to or during the methioninase
contacting step. A methionine starvation step may comprise the
use *in vitro* of methionine-deficient medium, i.e., medium with
low levels or no methionine, or the use *in vivo* of a methionine-
free diet. Methionine-free media are well known in the tissue
15   culture arts. An exemplary medium is Eagle's minimal essential
medium (lacking methionine and choline chloride) with nonessen-
tial amino acids, such as is available from GIBCO. Methionine
deficient amino acid diet foods are also commercially available,
including diet TD 92077 available from Teklad, Inc.
20   The time period for a methionine starvation step can vary
widely depending on the particular application, and is a time
period sufficient for cell culture, tissue or vascular
concentrations of methionine to decrease to a lower level, and
stop any further substantial decrease in level. Typical time
25   periods can be from about 6 hours to about 2 months, preferably
about 1 day to 2 weeks.
        Because methionine is an essential amino acid, it is to be
understood that many applications of the present methods can be
somewhat toxic to normal cells. However, as described herein,
30   normal and tumor cell metabolize the methionine precursor
homocysteine differently, such that homocysteine can supplement
methionine deficiencies in normal cells while not rescuing
methionine dependencies of tumor cells.
        Thus, in a related embodiment the invention contemplates a
35   method using a methionine-deficient nutrient (medium or diet)

which may be supplemented with a methionine precursor such as
homocysteine, or an analog thereof, used by normal cells to
provide necessary nutritional supplements. Homocysteine can be
added to nutrient medium at a concentration of about 5 to about
5   200 micromolar (uM), preferably about 10 to about 100 uM.

Preferred methionine precursors useful in this embodiment
include L-homocysteine-thiolactone, homocysteine, and 4-
methylthio-2-oxobutanoic acid.

Thus, in one embodiment, the invention comprises the step
10   of feeding a mammal with a methionine deficient diet for a time
period to deplete the intravascular concentrations of
methionine. The diet may optionally contain methionine
precursors as a methionine supplement. Alternatively, the
methionine precursor may be administered by injection or other
15   routes as is well known. Preferred daily dosages of
homocysteine as a dietary supplement are about 5 to about 1000
mg homocysteine per kilogram mammal body weight per day.

The routes of administration of the homocysteine are
typically the same as the methioninase to be added, and depend
20   on the target tissue for delivery of the supplement.

b.   Competitive Inhibitors of Methionine Utilizing Enzymes

Following the observations described in the Examples, it
has been further discovered that competitive inhibitors of
25   methionine-utilizing enzymes are useful to synergistically
increase the effectiveness of methionine depletion methods
described herein. Thus the invention contemplates the use of a
methionine starvation step that further comprises contacting the
tumor cells with an amount of a competitive inhibitor of
30   methionine for a time period sufficient to inhibit methionine
metabolism by a methionine-utilizing enzyme. Methionine-
utilizing enzymes useful for directing competitive inhibitors
include S-adenosyl methionine decarboxylase, methionine t-RNA
synthase and methionine adenosyltransferase.

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Preferably, the time period required to inhibit methionine
is the duration of the methionine starvation time period itself.

Competitive inhibitors of methionine compete with
methionine as a substrate for methionine-utilizing enzymes, and
5   therefor act to inhibit any normal metabolic effect that
endogenous methionine might produce by direct competition, i.e.,
inhibits methionine metabolism. Where the cell requires
methionine and the attendant metabolism of methionine, the
competitive inhibitor acts to reduce the metabolism of
10   methionine, necessitating higher methionine concentrations to
yield the same effect observed where no inhibitor is present.

A competitive inhibitor of methionine can be any methionine
derivative that functions as a classic competitive inhibitor.
Typical competitive inhibitors include alkyl derivatives of
15   methionine (i.e., alkylthionines), as where the methyl group of
methionine is replaced with an ethyl group (ethionine), a propyl
group (propthionine), a butyl group (buthionine), or a pentyl
group (penthionine).

Also contemplated as useful methionine competitive
20   inhibitors are cycloleucine and halogenated methionines. A
typical halogenated methionine is selected from the group
consisting of fluoromethionine, chloromethionine,
bromomethionine and iodomethionine. Thus a contemplated
competitive inhibitor of methionine is selected from the group
25   consisting of alkylthionine, cycloleucine and a halogenated
methionine, wherein said alkylthionine is not methionine.

An amount of a competitive inhibitor of methionine
effective to competitively inhibit methionine is an amount to
produce a reduction in the effective concentration of
30   methionine. This amount is typically a molar excess relative to
the methionine present, as shown in the Examples. Typically,
this amount of inhibitor is in the range of a 10 to 1000 fold
molar excess relative to the methionine concentration in the
medium where methionine is to be competed, preferably at least a
35   20 fold molar excess, and more preferably at least a 50 fold

molar excess. Where the inhibitor is to be used *in vivo*, the
dosage is typically about 5 - 30 mg per kg of animal body
weight, preferably about 25 mg/kg, as shown herein.

The amount of inhibitor required for effectiveness may vary
5   depending upon the amount of endogenous methionine present at
the time the inhibitor is administered. This relationship
between concentration of methionine and effective concentration
of inhibitor is demonstrated by the dosage titration results
shown in the Examples, and is defined by classic reaction rate
10   theory for competitive inhibitors. Typical amounts of inhibitor
are from about 10 uM to about 1 mM.

Furthermore, the amount of effective inhibitor can be
predetermined by the *in vitro* histoculture methods described
herein, to first establish the effective conditions in a
15   particular tumor tissue for methionine depletion by any of the
methods described herein, followed by a determination of the
effective concentration of the inhibitor.

The inhibition of methionine metabolism by use of a
competitive inhibitor of methionine can be monitored by a
20   variety of indicators, including the indicators described in the
Examples. These indicators include histoculture of a tissue to
measure DNA content as an indicator of cell cycle arrest, and
increased inhibition of growth of tumor tissue in a mouse fed a
methionine-free diet. Other indicators will be apparent to one
25   skilled in the art.

        c.   Synergistic Effect of Using Multiple Methionine
              Depletion Methods - Anti-Methionine Chemotherapy

In one embodiment, the present invention contemplates the
30   use of multiple methods for methionine depletion, generally
referred to as anti-methionine chemotherapy, to take advantage
of the synergy that occurs when two or more different methods
for methionine depletion are used.

Methionine depletion methods described herein include (1)
35   methionine starvation using methionine-free medium (*in vitro*) or

diet (_in vivo_), (2) exposure to methioninase to enzymatically
deplete endogenous methionine levels, (3) competitive inhibitors
of methionine to reduce the effective concentration of
endogenous methionine, particularly in combination with methods

5   (1) and (2) above, where methionine levels are already reduced,
and (4) use of methionine precursors, such as homocysteine
described herein, to increase the selectivity of any of the
other three methionine depletion methods for tumor cells.

Thus, anti-methionine chemotherapy methods described herein

10   for methionine depletion can be conducted by any of a variety of
combinations of the above identified methods, including (1) plus
(2), (1) plus (3), (2) plus (3), (1) plus (2) plus (3), and any
of these four combinations plus (4).

Particularly preferred for maximum depletion of methionine

15   and its resultant metabolites is the use of methionine-free diet
but containing methionine precursors, plus the use of a
competitive inhibitor of methionine and methioninase.

III. Enhancement of Anti-Mitotic Tumor Therapy

20   In another embodiment, the invention contemplates the use
of methionine-depletion methods described herein in methods for
increasing (enhancing) the potency and selectivity of
conventional chemotherapies, and preferably the selectivity of
antimitotic drugs used for cancer therapy.

25   The key purpose for use of methionine-depletion steps in
combination with conventional antimitotic therapies is to take
advantage of the fact that methionine depletion selectively and
specifically stops tumor cell proliferation prior to mitosis,
such that upon repleting the cell, tissue, medium or vasculature

30   with methionine, the static cells synchronously begin cell
cycling, into mitosis rendering the population of cells to be
uniformly proliferating and susceptible to the effects of cell-
cycle specific chemotherapy. To cause the tumor cells to enter
cell stasis, any cell cycle cytotoxic agent may be used. Such

35   agents are well known to those of ordinary skill in the art. In

a preferred embodiment, the cell-cycle cytotoxic agent is an anti-mitotic agent.

Thus, in a preferred embodiment, the present invention contemplates an anti-tumor chemotherapeutic method comprising the methionine-depleting step described herein to form static tumor cells followed by the additional steps of:

(a) contacting the static tumor cells with a cell cycle-inducing amount of methionine to initiate mitosis of the static tumor cells, thereby forming mitotic cells, and

(b) contacting the mitotic cells with an anti-mitotic agent in an amount sufficient to inhibit mitosis of the mitotic cells, thereby inhibiting tumor cell growth.

Methionine, methionine salts and functional equivalents thereof that function to initiate cell cycling of a static cell are referred to as cell cycle inducing agents, and can be used together or alternatively as the reagent for initiating cell cycling and mitosis in one or more static cells in the methionine-depleted tumor cell population.

A cell-cycle inducing amount of a cell cycle-inducing agent is an amount that initiates cell cycling in at least a few (10%), preferably a majority, and more preferably at least 90% of the static cells in the methionine-depleted tumor cell population. The initiation and extent of cell cycling can readily be measured by use of histological or metabolic markers. For methionine, a cell-cycle inducing amount is typically in the range of about 1 micromolar (uM) to about 2.5 millimolar (mM), and preferably about 10 to 250 uM. S-phase-specific drugs can similarly be used to attack any tumor cells still in S-phase when methionine is repleted.

Routes of contacting (administration) of the cell cycle inducing agent with the static tumor cells can vary but typically will be the same routes as used for administering methioninase or competitive inhibitors as described herein.

The timing and time periods for contacting the static tumor cells with a cell-cycle inducing agent can vary depending on the

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tumor, and other considerations that can be determined
empirically, such as by the histoculture methods described
herein. The cycle-inducing agent can be added prior to or
substantially simultaneously with the antimitotic agent. It may
5   be advantageous to add the inducer prior to the antimitotic
agent where it has been determined that the particular
antimitotic is fast acting, and cell cycle induction is slow.
These parameters may depend upon the particular tumor type,
tissue location, choice of antimitotic and the like.
10   Furthermore, optimization of variables such as timing, dosage
and drug choice can be readily carried out by a variety of
methods prior to *in vivo* administration of the therapeutic
methods. A preferred optimization procedure is to use the
exemplary histoculture assay system described herein.
15   The timing for contacting an anti-mitotic agent depends
upon the induction of cell cycling of the static tumor cells.
Typically, anti-mitotic agents can be contacted with the mitotic
cells at any time during cycling, and as soon as the cells
became mitotic. For some tumor cell populations, this event may
20   occur rapidly after addition of the inducer agent, allowing
simultaneous or near simultaneous addition of both inducer and
anti-mitotic agent to the static tumor cells. Alternatively,
the anti-mitotic may be added about 1 hour to 30 days after
inducer, but preferably within about one or two days of
25   induction.
          The anti-mitotic agent or other cell-cycle specific agent
used herein can be any agent exhibiting cell toxicity based
mechanisms directed at cell proliferation, mitosis or cell
cycling. Thus anti-mitotic agents can include any of a variety
30   of compounds which are anti-metabolites or which otherwise
exhibit toxicity for dividing (mitotic) cells. Preferred
clinical pharmacology of a anti-mitotic agent for use in the
present methods is inhibition of cell mitotic activity,
inhibition of nucleic acid synthesis, alkylating agents,
35   antibiotics, alkaloids, and the like antitumor agents. Insofar

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as the field of pharmacology is constantly advancing, it is to
be understood that the invention is to not be limited to
presently known anti-mitotic and other cell-cycle specific
agents, but rather to include the use of equivalents of known
5   compounds, newly discovered or developed compounds and the like
agents exhibiting the requisite activity.

Exemplary alkylating agents include cyclophosphamide (CTX;
cytoxan), chlorambucil (CHL; leukeran), cisplatin (CisP;
platinol) busulfan (myleran), melphalan, carmustine (BCNU),
10   streptozotocin, triethylenemelamine (TEM), mitomycin C, and the
like alkylating agents.

Exemplary anti-metabolites include methotrexate (MTX),
etoposide (VP16; vepesid) 6-mercaptopurine (6MP), 6-thioguanine
(6TG), cytarabine (Ara-C), 5-fluorouracil (5FU), dacarbazine
15   (DTIC), and the like anti-metabolites.

Exemplary antibiotics include actinomycin D, doxorubicin
(DXR; adriamycin), daunorubicin (daunomycin), bleomycin,
mithramycin and the like antibiotics.

Exemplary alkaloids include vinca alkaloids such as
20   vincristine (VCR), vinblastine, and the like.

Other antitumor agents include taxol and taxol derivatives,
the cytostatic agents glucocorticoids such as dexamethasone
(DEX; decadron) and corticosteroids such as prednisone,
nucleoside enzyme inhibitors such as hydroxyurea, amino acid
25   depleting enzymes such as asparaginase, and the like diverse
antitumor agents.

The synthesis and formulation of the above anti-mitotic
(cytotoxic) agents is well known, is described in a variety of
sources, and therefore will not be repeated here. Exemplary
30   sources for synthesis and formulations of the above agents
include Physician's Desk Reference, Barnhart, eds., Medical
Economics Company, Inc., Oradell, N.J., 1992; and Merck Index,
11th Edition, Merck & Co., 1989.

The use of cytotoxic agents described above in chemo-
35   therapeutic regimens is generally well characterized in the

cancer therapy arts, and their use herein falls under the same
considerations for monitoring tolerance and effectiveness and
for controlling administration routes and dosages, with some
adjustments. For example, the actual dosages of the cytotoxic
5   agents may vary depending upon the patient's cultured cell
response determined by using the present histoculture methods.
Generally, the dosage will be reduced compared to the amount
used in the absence of synchronized cell cycling, as shown by
the present disclosures.
10   Typical dosages of an effective cytotoxic agent can be in
the ranges recommended by the manufacturer, and where indicated
by *in vitro* responses or responses in animal models, can be
reduced by up to about one order of magnitude concentration or
amount. Thus, the actual dosage will depend upon the judgment
15   of the physician, the condition of the patient, and the
effectiveness of the therapeutic method based on the *in vitro*
responsiveness of the primary cultured malignant cells or
histocultured tissue sample, or the responses observed in the
appropriate animal models.
20

        IV. Gene Therapy
        In another embodiment, the invention contemplates the use
of a rDNA for expression of methioninase within the tissue or
lymphocytes surrounding the tissue to be treated according to
25   the methioninase contacting methods of the invention. To that
end, it is contemplated that the methioninase enzyme is
presented for the contacting step by the expression of a
regulatable methioninase expressing gene, thereby producing the
methioninase gene product.
30   As described earlier, there are a large variety of suitable
expression vectors presently known for expressing methioninase
which can be used. In one embodiment, the expression of the
methioninase gene is regulatable. Thus an expression vector has
a second nucleotide sequence operably linked to the first
35   methioninase encoding sequence which defines a means for

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regulating the expression of the methioninase gene. An
exemplary means is an inducible promoter, the preferred being
promoters that are tumor specific. Expression modules in which
the expression of the methioninase gene is controlled by a tumor
5   specific promoter are particularly useful for gene therapy as
herein described.

One class of inducible promoters are responsive to a
component that can be added to the culture medium, or readily
penetrate into a host cell containing the gene for expressing
10   the methioninase gene. Another class are tumor specific
promoters as described above, for example the carcinoembryonic
antigen (CEA) promoter.

Thus, the invention also contemplates a methionine
depleting method wherein the contacting of methioninase with a
15   population of tumor cells comprises the steps of:

(i)   introducing an expression vector into the tumor
cells or into tumor infiltrating lymphocytes or their bone
marrow precursors *in vivo* or *ex vivo*, wherein the expression
vector has a nucleotide sequence that encodes methioninase and
20   is capable of regulated expression of methioninase, and has a
nucleotide sequence that provides a means for regulating the
expression of methioninase, to form methioninase gene
transfected cells;

(ii)   contacting the tumor cells *in vivo* with the
25   methioninase gene transfected cells; and

(iii) expressing the methioninase-encoding nucleotide
sequence in the transfected cells, thereby producing
methioninase in the transfected cells and thereby contacting
tumor cells with the produced methioninase. By "introducing" is
30   meant any method known by those of ordinary skill in the art to
insert the expression vector into the target cells in a manner
wherein the gene contained within the expression vector may be
expressed by the target cell. The introduction can be performed
ex vivo or *in vivo*. Such "introducing" may be accomplished by,
35   for example, transfection, transformation, or viral infection.

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Transfection of tumor cells, either adherent cells or cells in suspension or *in vivo*, can be accomplished by a variety of known transfection methods. Thereafter, the transfected cell (if *ex vivo* tranfection is used) is reintroduced into the host
5   and allowed to locate in the native, relevant, vicinity of tissue to be treated, thereby contacting the transfected cells with the tumor cells to be treated. Introduction of methioninase to transfected cells can be accomplished by a variety of means, but generally requires the presence of a
10 selectable marker.

In one embodiments, the means for regulating the expression of the gene is an inducible promoter that is responsive to a reagent that can be added to the medium. Exemplary is the metallothionine promoter. Alternatively, the inducible promoter
15 can be a tumor specific promoter. Such a promoter acts to target methioninase expression to the tumor cell.

The cells to be transfected can be a sample of the tumor cells, or normal immune cells such as tumor infiltrating cells, such as tumor infiltrating lymphocytes or bone marrow precursors
20 of the bone marrow, such as hematopoietic stem cells or progenitor cells. The cells can be either *in vivo* or *ex vivo*.

Example 9 describes the isolation of a nucleic acid molecule encoding methioninase. The Example further describes the generation of expression vectors for use in batch
25 fermentation for producing methioninase. A skilled artisan can readily use the cloned methioninase gene, such as that provided in pAC-1, to produce expression cassette suitable for use in the above-described gene therapy.

30   C.   Therapeutic Compositions

In another embodiment, the present invention contemplates therapeutic compositions comprising a therapeutically effective amount of substantially isolated methioninase preferably recombinantly produced, together with a pharmaceutically
35 acceptable carrier.

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L-Methioninase (L-methionine-alpha-deamino-gamma-mercaptomethane-lyase or methioninase) is an enzyme that degrades methionine by deamination and dethiomethylation. Methioninase activity can be measured at least by measuring the
5   amount of alpha-ketobutyrate formed upon cleavage of methionine. One unit (U) of methioninase is defined as an amount of enzyme that produces 1 micromole of alpha-ketobutyrate per minute from methionine under the standard assay conditions described by Ito et al., J. Biochem., 79:1263-1272, 1976; and Soda, Analyt.
10   Biochem. 25:228-235, 1968.

Methioninase can be prepared from a variety of sources, including being isolated directly from bacteria cultures, or being expressed from a recombinant DNA molecule encoding a methioninase protein, such as described in Examples 9 and 12.

15   Bacterial sources of methioninase include Pseudomonas putida, Pseudomonas ovalis, and Aeromonas sp. and any other potential sources of methioninase. P. putida strains are commercially available from the ATCC and have accession numbers ATCC 8209 and ATCC 7955, respectively. The other bacteria are
20   generally available from the academic research community. Purification of methioninase has been described by a variety of methods. See, for example, Kreis et al., Cancer Res., 33:1862-1865, 1973; Tanaka et al., FEBS Letters 66:307-311, 1976; Ito et al., J. Biochem. 79:1263-1272, 1976; Nakayama et al., Agric.
25   Biol. Chem. 48:2367-2369, 1984; and Soda, Analyt. Biochem. 25:228-235, 1968. However, none of these methods results in a highly pure, endotoxin free methioninase. The present invention provide two methods that have been used to purify methioninase so that it is substantially endotoxin free.

30   A preferred methioninase has a specific activity of about 10 to about 50 units (U) per mg protein. Typical preparations of purified methioninase are described herein having a specific activity of about 10 to about 50 U/mg. In Example 12, methioninase prepared using the expression vector pAC-1 had a
35   specific activity of about 20.1 U/mg.

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A preferred methioninase is preferably substantially
isolated. By substantially isolated is meant that the enzyme is
at least 50% pure by weight, preferably at least 90% pure, and
more preferably at least 99% pure, or essentially homogeneous.

5   A preferred protein is essentially homogeneous when analyzed on
electrophoretic media such as polyacrylamide gel electrophoresis
(PAGE). Homogeneous on PAGE means only a single detectable
band.

A preferred methioninase is substantially free of

10   endotoxins, such as bacterial lipopolysaccharides, due to the
undesirable side effects associated with endotoxins when
physiologically contacted in a mammal, as by i.v. or i.p.
administration. By substantially free is meant less than about
10 nanograms (ng) endotoxin per milligram (mg) methioninase

15   protein, preferably less than 1 ng endotoxin per mg
methioninase, and more preferably less than 0.1 ng endotoxin per
mg methioninase. The assay of endotoxin is well known in the
therapeutic arts, and can be conducted by any of variety of
methods. A preferred method is described in the Examples.

20   A preferred methioninase is heat stable, so as to increase
its shelf life and effectiveness when utilized under conditions
of elevated temperatures, such as *in vivo* in an animal body. By
heat stable is meant that the enzyme can be exposed to 60
degrees Centigrade (60°C) for 10 minutes and retain 80%,

25   preferably retain 90%, and more preferably retain 95% of its
specific activity.

A preferred methioninase exhibits a serum half-life of at
least 5 hours, preferably 6 hours, and more preferably at least
7 hours.

30   Particularly preferred is methioninase prepared from P.
putida, or using an expression vector containing a DNA molecule
isolated from P. putida that encodes methioninase. P. putida
methioninase exhibits an apparent molecular weight when analyzed
on PAGE-SDS under denaturing conditions of about 43 kilodaltons.

A preferred method of purifying methioninase is described in the
Examples.

Thus a therapeutic composition comprises a physiologically
tolerable carrier together with substantially isolated

5   methioninase, dissolved or dispersed therein as an active
ingredient. In a preferred embodiment, the therapeutic
composition is not immunogenic when administered to a human
patient for therapeutic purposes.

One embodiment of the present invention features a

10   methioninase that is chemically modified comprising methioninase
conjugated to a polymer. Preferably, the methioninase is
substantially isolated and substantially endotoxin free. By
"chemically modified" is meant any form of methioninase that is
changed to a form that is different than the methioninase

15   purified from nature. Preferably, the methioninase is
chemically modified by linking the methioninase to a polymer
such as polyethylene glycol.

As used herein, the terms "pharmaceutically acceptable",
"physiologically tolerable" and grammatical variations thereof,

20   as they refer to compositions, carriers, diluents and reagents,
are used interchangeably and represent that the materials are
capable of administration to or upon a mammal or human without
the production of undesirable physiological effects such as
nausea, dizziness, gastric upset and the like.

25   The preparation of a pharmacological composition that
contains active ingredients dissolved or dispersed therein is
well understood in the art. Typically such compositions are
prepared as sterile injectables either as liquid solutions or
suspensions, aqueous or non-aqueous, however, solid forms

30   suitable for solution, or suspensions, in liquid prior to use
can also be prepared. The preparation can also be emulsified.
Particularly preferred are phospholipid and liposome
compositions as described herein. In addition, a therapeutic
amount of methioninase can be present in a ointment or on a

diffusible patch, such as a bandage, as to afford local delivery
of the agent.

The active ingredient can be mixed with excipients which
are pharmaceutically acceptable and compatible with the active
5   ingredient and in amounts suitable for use in the therapeutic
methods described herein. Suitable excipients are, for example,
water, saline, dextrose, glycerol, or the like and combinations
thereof. In addition, if desired, the composition can contain
minor amounts of auxiliary substances such as wetting or emulsi-
10   fying agents, pH buffering agents and the like which enhance the
effectiveness of the active ingredient.

The therapeutic composition of the present invention can
include pharmaceutically acceptable salts of the components
therein. Pharmaceutically acceptable salts include the acid
15   addition salts (formed with the free amino groups of the
polypeptide) that are formed with inorganic acids such as, for
example, hydrochloric or phosphoric acids, or such organic acids
as acetic, tartaric, mandelic and the like. Salts formed with
the free carboxyl groups can also be derived from inorganic
20   bases such as, for example, sodium, potassium, ammonium, calcium
or ferric hydroxides, and such organic bases as isopropylamine,
trimethylamine, 2-ethylamino ethanol, histidine, procaine and
the like.

Physiologically tolerable carriers are well known in the
25   art. Exemplary of liquid carriers are sterile aqueous solutions
that contain no materials in addition to the active ingredients
and water, or contain a buffer such as sodium phosphate at -
physiological pH value, physiological saline or both, such as
phosphate-buffered saline. Still further, aqueous carriers can
30   contain more than one buffer salt, as well as salts such as
sodium and potassium chlorides, dextrose, propylene glycol,
polyethylene glycol and other solutes.

Liquid compositions can also contain liquid phases in
addition to and to the exclusion of water, as described herein.
35   Exemplary of such additional liquid phases are glycerin,

vegetable oils such as cottonseed oil, organic esters such as
ethyl oleate, and water-oil emulsions, particularly the liposome
compositions described earlier.

A therapeutic composition contains an effective amount of
5   methioninase, typically an amount of at least 0.1 weight percent
of active protein per weight of total therapeutic composition,
and preferably is at least about 25 weight percent. A weight
percent is a ratio by weight of methioninase protein to total
composition. Thus, for example, 0.1 weight percent is 0.1 grams
10 of methioninase per 100 grams of total composition.

Insofar as a methioninase composition can be used *in vivo*
intravascularly, it is contemplated in one embodiment to
formulate a therapeutic composition for controlled delivery of
the methioninase, and optionally to shield the methioninase
15 protein from degradation and other phenomenon which would reduce
the serum half-life of therapeutically administered
methioninase.

Thus, in one embodiment, the invention contemplates
therapeutic compositions containing delivery vehicles such as
20 polymers, polymeric vehicles, particulates, latexes,
coacervates, ion-exchange resins, liposomes, enteric coatings,
mediators, bioadhesives, microcapsules, hydrogels, and the like
vehicles. Exemplary drug delivery vehicles including liposomes
are described at least by Tarcha in "Polymers For Controlled
25 Drug Delivery", CRC Press, Boca Raton, 1990.

The present invention further provides detailed methods for
purifying methioninase that provide superior yields and purity
when compared to other known methods. The preferred method for
purifying recombinant methioninase, particularly that produced
30 using a transformed host containing a high expression module
encoding methioninase, comprises the steps of:

a)   heating an extract of a transformed cell that contains
methioninase in aqueous buffers from about 40-60°C for about 1-
10 min., preferably 50°C for 1 min.;

   b)   centrifugation of the heated extract from about 10k to
20k rpm in a GS-3 rotor (Sorvall, Du Pont) for about 15 min. to
1 hour, preferably at about 13K rpm for about 30 min. at 4°C;

   c)   ultrafiltration of the supernatant using a filter of
5   about 50K to 100K pore size, preferably a Millipore
Pre/Scale:TFF PLHK 100 K 2.5 ft^2 cartridge using a 10mM potassium
phosphate buffer (pH8.3);

   d)   DEAE ion exchange chromatography in low ionic strength
(from about 10-50mM) KCl in a 10-20 mM potassium phosphate
10   buffer at about pH 7.0-7.6, and collecting fractions containing
methioninase eluted in a 40-200mM KCl gradient, preferably using
DEAE-Sepharose FF column;

   e)   a second DEAE ion exchange chromatography in medium
ionic strength (50-100mM) KCl in a 10-20 mM potassium phosphate
15   buffer at about pH 8.0-8.6, and collecting fractions containing
methioninase eluted in a phosphate buffer (pH 8.3) eluted in
100-200 mM KCl, preferably using DEAE-Sepharose FF column; and

   f)   contacting said fractions collected in step (e) with a
chromatography medium capable of absorbing endotoxin, and
20   collecting the eluant, thereby removing endotoxin from said
eluant to form endotoxin-free methioninase having at least 20
units methioninase activity per milligram protein and from 1-100
ng of endotoxin per mg protein, preferably using an Acticlean®
Etox column.

25   The cell extract is preferably prepared from a host cell
that has been altered to express high levels of recombinant
methioninase (from about 5-75% of total cellular protein)
Alternatively, an organism that naturally expresses methioninase
can be used as the starting material. For bacterial cell
30   extracts, the extracts are generally prepared by first
harvesting and washing bacterial cell cultures to form a cell
paste/pellet, depending upon whether harvesting is by
centrifugation or by hollow fiber filtration, which methods are
generally well known.

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The cells are then disrupted using conventional means. Preferably the cells are disrupted using a homogenizer, such as a cavitator-type homogenizer, for example, a Microfluidics Corp. Model #HC8000.

The resulting suspension is heated to precipitate selective proteins and other insoluble materials. Typical heating conditions are from about 45-60°C for 1-10 minutes. Preferred is a heating step of 50°C for 1 minute.

The heated extract is centrifuged to remove debris, and the supernatant is filtered and applied to DEAE ion-exchange chromatography medium in two steps as described above. Preferred adsorption and elution conditions are described in the Examples. Any of a variety of DEAE ion exchange column chromatography media can be used in these steps, and the choice of media is not to be construed as limiting. Commercial sources include Pharmacia Fine Chemicals, BioRad, and Sigma.

Thereafter, endotoxin is removed to produce a protein having acceptable levels of endotoxin as recited earlier. The endotoxin removal step can be carried out in any of a variety of means, as are well known, and typically involve contacting the protein in solution with a chromatography medium capable of adsorbing endotoxin, and yielding a chromatography medium eluant which contains endotoxin-free protein. The preferred commercial reagent for use in removing endotoxin is Acticlean® Etox.

D.   Chemically Modified Methioninase

Methioninase may be conjugated to a polymer with the purpose of extending its half-life and decreasing its immunogenicity or antigenicity.

There is the possibility of an allergic response in over-sensitive individuals. On the other hand, anti-methioninase antibodies may shorten the half - life of methioninase.

Various approaches have been taken in attempts to solve the problem of antigenicity of polypeptides and proteins. Coupling of polymers such as polyethylene glycol to proteins is one of

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the approaches to reducing protein antigenicity. The present
invention relates to chemical modification of methioninase
produced by a novel and effective method in which purified
methioninase is coupled with polyethylene glycol (PEG) under

5   conditions which maintain the activity of the enzyme. The PEG-
methioninase of this invention has an extended half life and no
apparent immunogenicity.

The novel methioninase conjugated to polyethylene glycol
("PEG-methioninase" or "PEGylated methioninase") exhibits a high

10   methionine depletion activity, an extended half-life, and low
immunogenicity. Thus PEG-methioninase provides a novel means of
inhibiting tumor growth while maintaining stability, increasing
its serum half-life, decreasing immunogenicity and decreasing
toxicity.

15   The present invention of PEG - methioninase which is
stable, effective in methionine depletion with a long half -
life and is non - immunogenic, may be utilized as a safe and
effective anti - tumor agent for multiple dosing. PEG -
methioninase may be stored in a liquid formulation of 0.12 M

20   sodium chloride in 10 mM sodium phosphate (pH 7.2) at - 70°C,
4°C and at room temperature without loss of activity.

Importantly, the PEG - methioninase of this invention is
non - immunogenic which renders it particularly desirable for
the treatment of cancer patients who are sensitive to

25   methioninase, and require repeated dosing. Those of ordinary
skill in the art will recognize that as with methioninase, the
PEG-methioninase may be provided in a variety of formulations.

A preferred formulation of the purified endotoxin - free
methioninase is in 0.12 M sodium chloride in 10 mM sodium

30   phosphate buffer (pH 7.2) at a concentration between 0.06 M and
0.2 M. The activity is approximately 10 units/mg.

The PEGylated methioninase may be tested by means well
known to those of ordinary skill in the art. For example, in
vivo testing may be used to determine the pharmacology, safety

and functional characteristics of PEG- methioninase produced by
the method of the invention.

Such tests may include determination of acute toxicity,
pharmacokinetics of PEG - methioninase, depletion of methionine
5   in serum and evaluation of immunogenicity of PEG - methioninase.

Purified endotoxin-free PEG - methioninase is injected into
the tail-vein of mice and the blood samples are collected every
two hours. The levels of methioninase are measured by the
activity assay. The levels of methionine are measured with HPLC
10   after methionine derivitization.

Acute toxicity studies may be performed in any suitable
experimental animals such as those described in Examples 5 and
15. In mice, 10 - 100 units / 1 - 10 mg of PEG - methioninase
are injected into the tail-vein. Vital signs and visual
15   observations are recorded. The blood samples are collected
before the injection and every two hours after injection. Both
the activity of methioninase and the levels of methionine are
measured. The functions of both kidney and liver function are
also measured. The presence or absence of toxic effects on
20   tissues such as lung, kidney, liver and brain are determined by
standard techniques. The blood and bone marrow are analyzed as
well.

In another embodiment, the methioninase is conjugated to a
polymer resulting in a substantially nonimmunogenic composition
25   and also resulting in an increase in the half-life of
methioninase activity _in vivo_.

In one embodiment the methioninase is chemically modified
by conjugation to a polymer.

In another embodiment the methioninase is chemically
30   modified by conjugation to a polyalkylene oxide. Examples of
polyalkylene oxides include, but are not limited to,
polyethylene oxide, polypropylene oxide, copolymers of ethylene
oxide, and copolymers of propylene oxide.

In a preferred embodiment the methioninase is chemically
35   modified by conjugation to polyethylene gylcol.

In another preferred embodiment the methioninase conjugated
to polyethylene glycol is substantially free of endotoxin.

The invention also provides a pharmaceutical composition
comprising a therapeutically effective amount of methioninase

5   conjugated to a polymer. The polymer may be a polyalkylene
oxide, for example, but not limited to, polyethylene oxide,
polypropylene oxide, copolymers of ethylene oxide, and
copolymers of propylene oxide.

In a preferred embodiment is provided a pharmaceutical

10   composition comprising a therapeutically effective amount of
methioninase conjugated to polyethylene glycol. In another
preferred embodiment the pharmaceutical composition comprises a
therapeutically effective amount of methioninase that is
substantially free of endotoxin and is conjugated to

15   polyethylene glycol. Such composition can be prepared from
material isolated from organisms that naturally produce
methioninase or preferably, from a host that has been altered to
express methioninase.

Also provided is a method for producing endotoxin-free

20   methioninase which is conjugated to a polymer by coupling
endotoxin-free methioninase which is conjugated to a polymer by
coupling endotoxin-free methioninase with a polymer to form a
substantially nonimmunogenic chemically modified endotoxin free
methioninase.

25   In a preferred embodiment the method includes coupling the
methioninase to polyethylene glycol.

In a further preferred embodiment the coupling is performed
by reacting endotoxin-free methioninase with methoxy
polyethylene glycol succinimidyl carbonate.

30   Also provided is a method of treating a patient having a
tumor comprising administering a therapeutically effective
amount of methioninase.

In a preferred embodiment the methioninase is substantially
free of endotoxin.

In a further preferred embodiment the methioninase is conjugated to a polymer.

In a further embodiment the polymer is a polyalkylene oxide.

5   In a further preferred embodiment the methioninase is conjugated to polyethylene glycol.

E.   Formulations of recombinant methioninase

The present invention further provides methioninase in
10   lyophilized or crystalline form. In detail, it has been observed that methioninase can readily be lyophilized or crystallized using art known methods. The resulting preparation of methioninase, crystallized or lyophilized forms, were found to be highly stable, readily hydratable, and remained highly
15   active following rehydration.

A variety of art known methods can be used to obtain methioninase in crystallized or lyophilized form. In the examples, lyophilization and crystallization of methioninase were performed using a Verdis, Freeze mobile 24, at 100
20   millibar, -80°C for 72 hours. A skilled artisan can readily adapt other art known procedures for use in producing lyophilized or crystallized forms of methioninase.

F   DNA Segments and Vectors
25   I. Methioninase-Coding DNA Molecules

It has been found that by operably linking an isolated DNA molecule encoding methioninase to a promoter, particularly an RNA polymerase promoter such as the T7 RNA polymerase promoter, recombinant methioninase can be expressed at levels from about
30   5-75% of total cellular protein, when introduced into an appropriate host cell. Accordingly, the invention provides high-level expression modules that express high levels of recombinant methioninase when introduced into a host under appropriate conditions.

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As used herein, a high-level expression module, or an expression module of the present invention, refers to a nucleic acid molecule that contains one or more expression control elements that direct the transcription and translation of an
5   operably linked nucleotide sequence that encodes methioninase. The expression module can be an isolated nucleic acid molecule or can be present in a vector (described below).

The expression modules of the present invention contain control elements that direct the production of recombinant
10   methioninase such that the recombinant methioninase produced represents from about 5-75% of total cellular protein, preferably more than 10% of total cellular protein. The preferred expression control elements are RNA polymerase promoters, the most preferred being the T7 RNA polymerase
15   promoter. Other examples of RNA polymerase promoters include, but are not limited to, the Tac and Trc promoters.

A promoter is an expression control element formed by a DNA sequence that permits binding of RNA polymerase and transcription to occur. Promoter sequences compatible with a
20   particular host system are known in the art and are typically provided in a plasmid vector containing one or more convenient restriction sites. Typical of such plasmids vectors are those containing the T7 RNA polymerase promoter, pT7 and pET that are available from a variety of sources such as commercial suppliers
25   and the American Type Culture Collection.

The expression modules of the present invention further comprise a nucleic acid sequence that encodes methioninase. As used herein, a nucleic acid sequence is said to encode methioninase when the transcription and translation of the
30   nucleic acid molecule comprising the sequence results in the production of a protein having methioninase activity.

L-Methioninase (L-methionine-alpha-deamino-gammamercaptomethane-lyase or methioninase) is an enzyme that degrades methionine by deamination and dethiomethylation.
35   Methioninase activity can be measured at least by measuring the

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amount of alpha-ketobutyrate formed upon cleavage of methionine.
One unit (U) of methioninase is defined as an amount of enzyme
that produces 1 micromole of alpha-ketobutyrate per minute from
methionine under the standard assay conditions described by Ito

5   *et al.*, J. Biochem., 79:1263-1272, 1976; and Soda, Analyt.
Biochem. 25:228-235, 1968.

The methioninase-encoding nucleic acid sequence can
comprise an unaltered sequence obtained from an organism that
naturally produces recombinant methioninase, or can comprise a

10   sequence obtained from an organism that naturally produces
methioninase that has been altered to contain one or more
nucleic acid or amino acid substitutions, deletions or
additions.

The methioninase-encoding nucleic acid molecule, whether

15   altered or unaltered, can be derived from any organism that
naturally produces methioninase. The preferred source of the
methioninase-encoding nucleic acid molecule is *Pseudomonas
putida*. Example 9 discloses the isolation and sequencing of a
methioninase-encoding nucleic acid molecule from *P. putida*.

20   Other preferred sources for a methioninase-encoding nucleic acid
molecule include, but are not limited to, *Trichomonas vaginalis*,
Nippostrongylus brasiliensis, and *Fusobacterium sp.*

The complete coding sequence for methioninase can be
obtained from a variety of sources, especially those recited

25   above, using a variety of methods. The isolation of
methioninase-encoding nucleic acid molecules from an organism
other than *P. putida* is greatly facilitated by the amino acid
and nucleic acid sequences provided in Figure 8.

Specifically, a skilled artisan can readily use the nucleic

30   acid sequence provided in Figure 8 to prepare pairs of
oligonucleotide primers for use in a polymerase chain reaction
(PCR) to selectively amplify a methioninase-encoding nucleic
acid molecule from methionine expressing organisms. The
preferred PCR primer pairs based on the sequence provided in

35   Figure 8 are:

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5'-GCCGGTCTGTGGAAT*AAGCT*-3' (Sense)

5'-CCAG*GGTCGA*CTCCAGCGCC-3' (Antisense)

A preferred PCR denature/anneal/extend cycle for using the above
PCR primers is as follows: first denaturation at 95°C for 10
minutes, then 5 cycles of denaturation at 94°C for 30 seconds,
annealing at 60°C for 30 seconds, and extension at 72°C for 2
minutes; then 25 cycles of denaturation at 94°C for 30
seconds, 60°C for 30 seconds, then extension at 72°C for 1.5
minutes; then final extension at 72°C for 10 minutes. The PCR
amplified products are two bands of which the 1365 bp band was
collected, and purified as the insert ONCase-1 DNA.

Alternatively, a fragment of the nucleotide sequence Figure
8 can be used as a probe to isolate DNA encoding methioninase
from organisms other than *Pseudomonas putida* using art-known
methods. Oligomers containing approximately 18-20 nucleotides
(encoding about a 6-7 amino acid stretch) are prepared and used
to probe genomic DNA libraries to obtain hybridization under
conditions of sufficient stringency to eliminate false positives
using procedures well known in the art. (See Sambrook *et al.*
Molecular Cloning, Cold Spring Harbor Press 1989)

DNA segments (i.e., synthetic oligonucleotides) that are
used as probes or specific primers for the polymerase chain
reaction (PCR), as well as gene sequences encoding methioninase,
can readily be synthesized by chemical techniques, for example,
the phosphotriester method of Matteucci, *et al.*, (J. Am. Chem.
Soc. 103:3185-3191, 1981) or using automated synthesis methods.
In addition, larger DNA segments can readily be prepared by well
known methods, such as synthesis of a group of oligonucleotides
that define the DNA segment, followed by hybridization and
ligation of oligonucleotides to build the complete segment.

In addition to PCR and DNA probe based methods, DNA
molecules encoding methioninase can be isolated using polyclonal
antiserum or monoclonal antibodies raised against peptide

fragments of Figure 8 that are predicted as being immunogenic.
Such antibodies can be used to probe an expression library
generated from a given organism, such as a lambda gt11 library,
to obtain DNA molecules encoding methioninase from an organism
5   other the *P. putida*.

In one embodiment, the methioninase-encoding nucleic
acid molecule is obtain, a skilled artisan can readily employ
random or site specific mutagenesis procedures to alter the
methioninase-encoding sequence so as to increase the level of
10   expression or to substitute, add, or delete one or more amino
acids from the encoded methioninase.

In one embodiment, the methioninase-encoding sequence is
altered so as to increase the level of expression of the
recombinant methioninase in a given host cell without changing
15   the amino acid sequence of the encoded methioninase. Increased
expression of recombinant methioninase in a particular host can
be obtained by altering one or more of the codons present in the
nucleic acid molecule so that the resulting codons are ones that
are more frequently used by the host organism to encode a
20   particular amino acid. Altering a nucleotide sequence to
contain preferred codons can be accomplished using art known
procedures such as site directed mutagenesis or by synthesizing
a nucleic acid molecule containing the preferred codons.

In addition to alterations that affect expression,
25   methioninase-encoding nucleic acid molecules can be altered so
as to facilitate purification of the resulting protein. For
example, as disclosed in the Examples, by altering either the
amino or carboxy terminus of the recombinant methioninase so as
to add a polyhistidine stretch, Ni^{++} sepharose can be used to
30   purify the resulting fusion protein.

The methioninase-encoding sequence can also be altered to
introduce changes in the amino acid sequence of the encoded
methioninase, so as to add, substitute, or delete one or more
amino acid residues. The resulting recombinant methioninase
35   will preferably contain alterations that result in recombinant

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methioninase with better biological or physiological properties
such as increased activity, decreased K_m, decreased
immunogenicity, or increased serum half life. Such altered
forms can be rationally designed or randomly generated.

5   An alteration is said to be rationally designed when the
alteration is specifically chosen based on the amino acid
sequence of the starting and resulting proteins and a desired
physiological property. For example, one type of rationally
designed alteration is to replace hydrophobic amino acids with

10   less hydrophobic residues to increase solubility. The preferred
method for generating rationally designed alterations is site
direct mutagenesis using a mismatched PCR primer extension
method.

       Alterations are said to be randomly generated when the

15   alteration is not rationally selected. Random mutagenesis
techniques, such as chemical mutagenesis, PCR shuffling and
linker scanning mutagenesis, generate a large variety of random
and non-specific alterations in a given protein encoding
sequence. Such methods can be used to radically alter the

20   methioninase-encoding nucleic acid molecule.

       Altered forms of recombinant methioninase generated in this
fashion are then screened for desired properties using a variety
of art known methods. The choice of selection method employed
will be dependent of the host, vector, and mutagenesis methods

25   employed as well as the properties that are selected for.

       The present invention further provides vectors containing
one or more of the expression modules of the present invention.
Vectors are DNA molecules that are capable of autonomous
replication within a host. Vectors can contain an episomal

30   origin of replication derived from a naturally occurring
plasmid, a genomic origin of replication, or can be derived from
a viral genome. The choice of the vector to which an expression
module of the present invention is inserted depends directly, as
is well known in the art, on the functional properties desired,

35   e.g., protein expression, and the host cell to be transformed.

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In one embodiment, the vector includes a prokaryotic replicon. Prokaryotic replicons such as the ColEl replicon, are well known in the art and can readily be employed in combination with an expression module of the present invention. In

5   addition, the vector may include a gene encoding a selectable marker such as a drug resistance.

Eukaryotic expression vectors can also be used in combination with an expression module of the present invention. Eukaryotic cell expression vectors are well known in the art and

10   are available from several commercial sources. Typical of such vectors are PSVL and pKSV-10 (Pharmacia), pBPV-1/pML2d (International Biotechnologies, Inc.), pTDT1 (ATCC, #31255), the vector pCDM8 described herein, and the like eukaryotic expression vectors. High level expression vectors can further

15   be generated using insect cell expression systems such as a bacculovirus based vector system.

In general terms, the generation of a high expression module encoding methioninase typically involves the following:

First, a DNA is obtained that encodes methioninase. If the

20   sequence is uninterrupted by introns, as expected from a bacterial source, it is suitable for expression in any host. This sequence may be altered to be in a readily excisable and recoverable form by inserting sequences containing one or more restriction endonuclease sites at regions flanking the

25   methioninase-encoding sequence.

The excised or recovered coding sequence is then placed in operable linkage with a high expression control element, preferably in a replicable expression vector. The expression module or vector is then used to transform a suitable host and

30   the transformed host is cultured under conditions to effect the production of the recombinant methioninase. Optionally the recombinant methioninase is isolated from the medium or from the cells; recovery and purification of the protein may not be necessary in some instances, where some impurities may be

35   tolerated.

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Each of the foregoing steps can be done in a variety of
ways. For example, the desired coding sequences may be obtained
from genomic fragments and used directly in appropriate hosts.
The constructions of expression vectors that are operable in a
5   variety of hosts are made using two or more appropriate
replicons and control elements. Suitable restriction sites can,
if not normally available, be added to the ends of the coding
sequence so as to provide an excisable gene to insert into these
vectors.

10

II.   Transformed Host Cells Expressing High Levels of
       Recombinant methioninase
The present invention further provides host cells
transformed with an expression module or vector of the present
15   invention so as to produce from about 5-75% of total cellular
protein as recombinant methioninase, preferably more than about
10% of total cellular protein. The host cell can be either a
prokaryotic or a eukaryotic host.
Any prokaryotic host can be used to express the high-level
20   methioninase-encoding modules of the present invention. The
preferred prokaryotic host is E. coli. In the Examples that
follow, the DH5α and BL21(DE3) strains of E. coli were used.
Preferred eukaryotic host cells include insect cells, yeast
cells and mammalian cells, preferably insect cells such as SP6
25   and vertebrate cells such as those from a mouse, rat, monkey or
human fibroblastic cell line. Other preferred eukaryotic host
cells include Chinese hamster ovary (CHO) cells available from
the ATCC as CCL61, NIH Swiss mouse embryo cells NIH/3T3
available from the ATCC as CRL 1658, baby hamster kidney cells
30   (BHK), and the like eukaryotic tissue culture cell lines.
Transformation of an appropriate host with a high-level
expression recombinant module of the present invention is
accomplished by well known methods that typically depend on the
type of host and vector used. With regard to transformation of
35   prokaryotic host cells, electroporation or salt treatment of the

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host cells is preferred, for example, see Cohen *et al.*, <u>Proc.</u>
<u>Natl. Acad. Sci. USA</u> <u>69</u>:2110, 1972; and Maniatis *et al.*,
<u>Molecular Cloning, A Laboratory Mammal</u>, Cold Spring Harbor
Laboratory, Cold Spring Harbor, NY (1982).

5   With regard to transformation of eukaryotic cells,
electroporation or the use of a cationic lipid is preferred, for
example, see Graham *et al.*, <u>Virol.</u> <u>52</u>:456, 1973; and Wigler *et*
al., <u>Proc. Natl. Acad. Sci. USA</u> <u>76</u>:1373-76, 1979.

        Successfully transformed cells, i.e., cells that contain an
10   expression module of the present invention, can be identified by
well known techniques. For example, cells resulting from the
introduction of an expression module of the present invention
can be cloned to produce single colonies. Cells from those
colonies can be harvested, lysed and their DNA content examined
15   for the presence of the rDNA using a method such as that
described by Southern, <u>J. Mol. Biol.</u>, <u>98</u>:503, 1975, or Berent *et*
al., <u>Biotech.</u> <u>3</u>:208, 1985. However, as described below, the
present invention further provides a rapid screening method to
identify transformants which express high levels of recombinant
20   methioninase.

        III.   <u>Identification of Hosts Expressing High Levels</u>
               <u>of Recombinant methioninase</u>
        The present invention further provides methods of
25   identifying a transformed host cell which produces recombinant
methioninase at levels from about 5-75% of total cellular
protein. Specifically, it has been observed that transformed
host cells expressing from about 5-75% of total cellular protein
as recombinant methioninase, have a distinct and observable pink
30   color. This is particularly pronounced when *E. coli* is used as
the host.

        To identify a transformed host cell expressing high levels
of recombinant methioninase, a transformed cell is grown on or
in a media under conditions in which the recombinant
35   methioninase is expressed and that allows visual inspection of

the growing cells. The growing cells or colonies are examined
and selected based on the displaying of a pink color.

A variety of culture/growth conditions can be employed to
grow transformed host cells for selection using the present
5   methods. The components of the growth medium will depend on the
nutritional requirements of the host/vector system employed and
inspection system used to identify the pink color associated
with high levels of recombinant methioninase expression and
thereby allowing isolation of a high-level expression clone.
10   The preferred medium is a solid medium onto which the
transformed host cells can be plated and grown as isolated
colonies, each of which is derived from single host. The
preferred method of identifying high-level expression clones is
visual inspection of growing colonies.

15

IV. Production of Recombinant Methioninase Using a
High-Level Expression Module

The present invention further provides methods for
producing recombinant methioninase. Specifically, recombinant
20   methioninase can be produced at commercially significant levels
using a host transformed with one or more of the high-level
expression modules of the present invention. Such a transformed
host will express recombinant methioninase at a level from about
5-75% of total cellular protein. Using the hosts of the present
25   invention, a skilled artisan can readily produce recombinant
methioninase for use in a variety of diagnostic and therapeutic
methods using art known methods.

The preferred method for purifying recombinant methioninase
produced using a transformed host containing a high expression
30   module encoding methioninase, or from a host that naturally
produces methioninase, comprises the steps of:

a)   heating an extract of a transformed cell that contains
methioninase in aqueous buffers from about 40-60°C for about 1-10
min., preferably 50°C for 1 min.;

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b)   centrifugation of the heated extract from about 10k to
20k rpm in a GS-3 rotor (Sorvall, Du Pont) for about 15 min. to
1 hour, preferably at about 13K rpm for about 30 min. at 4°C;

c)   ultrafiltration of the supernatant using a filter of
5   about 50K to 100K pore size, preferably a Millipore
Pre/Scale:TFF PLHK 100 K 2.5 ft^2 cartridge using a 10mM potassium
phosphate buffer (pH8.3);

d)   DEAE ion exchange chromatography in low ionic strength
(from about 10-50mM) KCl in a 10-20 mM potassium phosphate
10   buffer at about pH 7.0-7.6, and collecting fractions containing
methioninase eluted in a 40-200mM KCl gradient, preferably using
DEAE-Sepharose FF column;

e)   a second DEAE ion exchange chromatography in medium
ionic strength (50-100mM) KCl in a 10-20 mM potassium phosphate
15   buffer at about pH 8.0-8.6, and collecting fractions containing
methioninase eluted in a phosphate buffer (pH 8.3) eluted in
100-200 mM KCl, preferably using DEAE-Sepharose FF column; and

f)   contacting said fractions collected in step (e) with a
chromatography medium capable of absorbing endotoxin, and
20   collecting the eluant, thereby removing endotoxin from said
eluant to form endotoxin-free methioninase having at least 20
units methioninase activity per milligram protein and from 1-100
ng of endotoxin per mg protein, preferably using an Acticlean®
Etox column.

25   The cell extract is preferably prepared from a host cell
that has been altered to express high levels of recombinant
methioninase (from about 5-75% of total cellular protein). For
bacterial cell extracts, the extracts are generally prepared by
first harvesting and washing bacterial cell cultures to form a
30   cell paste/pellet, depending upon whether harvesting is by
centrifugation or by hollow fiber filtration, which methods are
generally well known.

The cells are then disrupted using conventional means.
Preferably the cells are disrupted using a homogenizer, such as

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a cavitator-type homogenizer, for example, a Microfluidics Corp.
Model #HC8000.

The resulting suspension is heated to precipitate selective
proteins and other insoluble materials. Typical heating
5   conditions are from about 45-60°C for 1-10 minutes. Preferred is
a heating step of 50°C for 1 minute.

The heated extract is centrifuged to remove debris, and the
supernatant is filtered and applied to DEAE ion-exchange
chromatography medium in two steps as described above.
10 Preferred adsorption and elution conditions are described in the
Examples. Any of a variety of DEAE ion exchange column
chromatography media can be used in these steps, and the choice
of media is not to be construed as limiting. Commercial sources
include Pharmacia Fine Chemicals, BioRad, and Sigma.
15   Thereafter, endotoxin is removed to produce a protein
having acceptable levels of endotoxin as recited earlier. The
endotoxin removal step can be carried out in any of a variety of
means, as are well known, and typically involve contacting the
protein in solution with a chromatography medium capable of
20 adsorbing endotoxin, and yielding a chromatography medium eluant
which contains endotoxin-free protein. The preferred commercial
reagent for use in removing endotoxin is Acticlean® Etox.

G.   Use of Methioninase for Prevention of
25   Hyperhomocysteinemia-associated Cardiovascular Disease
Another aspect of the invention is the use of methioninase
for homocysteine depletion therapy.

Hyperhomocysteinemia has been associated with various types
of cardiovascular diseases. The first association of these
30 diseases with homocysteine was made by McCully in 1969.
(McCully, KS, Am. J. Pathol. 56: 111-28, 1969) McCully found a
link between elevated plasma homocysteine concentrations and
arteriosclerotic diseases. A recently concluded study of 1,041
people from the Framingham Heart Study found that elevated
35 plasma levels of homocysteine leads to an increased risk of

arteriosclerosis (Selhub, J. et al., N. Engl. J. Med. 32:286-91,
1995). Other studies have linked even moderate
hyperhomocysteinemia to peripheral vascular, cerebrovascular and
coronary heart disease. (Kang, S. et al., Annu. Rev. Nutr.

5   12:279-98, 1992). For example, fasting homocysteine
concentrations in patients with vascular disease are 31% higher
than in normal subjects. Plasma homocysteine concentrations 12%
above the upper limit of normal were associated with the 3.4-
fold increase in risk for myocardial infarction. (Stampfer, M.

10   et al., JAMA 268:877-81, 1992). Studies of homocysteine
metabolism have found that in 20 case control and cross-
sectional studies of over 2000 subjects, show that patients with
stroke and other cardiovascular blood diseases have higher blood
levels of homocysteine than subjects with no cardiovascular

15   diseases. This is in contrast with the fact that most patients
with myocardial infarction have normal cholesterol levels. A
striking result was found in the Physicians Health Study, where
it was shown prospectively that where blood was drawn before
cardiovascular disease was diagnosed 271 men who later had

20   myocardial infarctions had significantly higher mean base line
levels of homocysteine than matched controls who did not have
myocardial infarctions (Ueland, P. et al., Cardiovascular
Disease Hemostasis and Endothelial Function, New York: Marcel
Dekler; 183-236, 1992). Although a variety of conditions can

25   lead to elevated homocysteine levels, the relation between high
levels of homocysteine and vascular disease is present
regardless of the underlying metabolic cause. In a direct
study, vascular lesions were induced in baboons and the baboons
were infused with homocysteine for three months (Ueland, P., et

30   al, supra). Hyperhomocysteinemia may be diagnosed by oral
administration of methionine followed by the measurement of the
levels of homocysteine in the patients. Abnormal homocysteine
plasma concentrations after this oral methionine challenge is
twelve times more present in patients with arteriosclerotic

35   disease than in normal subjects (Ueland, et al., supra). Plasma

homocysteine levels can be easily and rapidly measured by HPLC
assays. Studies suggest that 40% of the population may have
elevated homocysteine levels and be at risk for cardiovascular
disease (Stampfer, M. and Malinow, M., New Engl. J. Med.
5   332:326-329 1995). Hyperhomocysteinemia can have acute effects
in inducing arteriosclerotic disease and putting individuals at
risk for myocardial infarction and cerebrovascular disease.
Acute medical intervention is thus indicated for a large
fraction of individuals at risk for cardiovascular disease. The
10 methioninase of the present invention may be used as the therapy
of choice for acute intervention to immediately lower
homocysteine levels in individuals at risk. Methioninase
catalyzes two reactions, the cleavage of both the nitrogen-
carbon bond and gamma-carbon-sulfar bond not only in methionine
15 but also in homocysteine (Hoffman, Bioch. et Biophys. Acta,
Reviews on Cancer, 738:49-87, 1984). Figure 7 illustrates the
metabolic cycle of homocysteine and methionine metabolism. The
vitamins B-12, B-6, and folate influence the "left" side of the
cycle illustrated in the figure and will be helpful in the
20 maintenance of normal homocysteine levels in some individuals
after methioninase treatments. However, the "right" side of the
cycle is independent of the levels of these vitamins. In the
right side of the cycle the presence of methionine can lead to
excess homocysteine levels via elevated transmethylation
25 reactions which may lead to cancer as well as to
arteriosclerotic disease. For individuals with abnormalities in
the right side of the cycle, maintenance as well as acute use of
methioninase may be necessary to maintain normal levels of
homocysteine. For cardiovascular disease, the substrate
30 specificity of methioninase for both methionine and homocysteine
is critical: methioninase will lower both the methionine level
which is a precursor of homocysteine and will also lower the
homocysteine level directly. Previous work in the area has only
suggested the use of vitamins B-12, B-6 and folate as therapy to
35 lower hyperhomocysteinemia. As shown in Figure 7, merely

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providing these vitamins will not rectify any abnormalities in
the right side of the cycle and may not decrease homocysteine in
individuals with abnormalities in the right side of the cycle.
The present invention includes a method of treating patients

5   having such cardiovascular diseases with methioninase.

In one aspect, a method is provided of treating a patient
having a cardiovascular disease comprising administering to the
patient a therapeutically effective amount of methioninase.

A therapeutically effective amount of methioninase for

10   homocysteine depletion is a predetermined amount calculated to
achieve the desired level of homocysteine depletion so as to,
for example, reduce the risk of arteriosclerosis.

Thus, the dosage ranges for the administration of
methioninase of the invention are those large enough to produce

15   the desired effect in which, for example, the risk of, or level
of, arteriosclerosis, are reduced. The dosage should not be so
large as to cause adverse side effects, such as hyperviscosity,
syndromes, pulmonary edema, congestive heart failure, and the
like. Generally, the dosage will vary with the age, condition,

20   sex and extent of the disease in the patient and can be
determined by one of skill in the art.

The dosage can be adjusted by the individual physician in
the event of any complication.

A therapeutically effective amount of a methioninase of

25   this invention is typically an amount such that when
administered in a physiologically tolerable composition is
sufficient to achieve a intravascular (plasma) or local
concentration of from about 0.001 to about 100 U per ml,
preferably above about 0.1 U, and more preferably above 1 U

30   methioninase per ml. Typical dosages can be administered based
on body weight, and are in the range of about 5-1000 U/kilogram
(kg)/day, preferably about 10-50 U/kg/day, and more preferably
about 20-40 U/kg/day.

In preferred methods, a methioninase used is substantially

35   free of endotoxin, as discussed in detail above. Particularly

preferred is the use of methioninase produced herein that is
substantially free of endotoxin from a recombinant source.

Methods of administration known to those of ordinary skill
in the art may be employed, and are described herein.

5   In another aspect of the invention a patient with a
cardiovascular disease is treated by administration of a
therapeutically effective amount of a methioninase which is
substantially free of endotoxins and is conjugated to a polymer.
In a preferred aspect, the polymer is polyethylene glycol. In

10   another preferred aspect of the invention the cardiovascular
disease is arteriosclerosis. In another aspect of the invention
methioninase is administered to patients with extracranial
carotid artery stenosis, peripheral vascular, cerebrovascular,
coronary heart disease, and occlusive vascular disease.

15   In another aspect of the invention is provided a method of
treating a patient having hyperhomocysteinemia by administering
to the patient a therapeutically effective amount of
methioninase. The methioninase may be substantially free of
endotoxins and the methioninase may be conjugated through a

20   polymer such as polyethylene glycol.

The invention also provides a method of lowering
homocysteine levels in a patient comprising the step of
administering to the patient a therapeutically effective amount
of methioninase wherein the methioninase is substantially free

25   of endotoxins. In another aspect of the invention a method is
provided of preventing cardiovascular disease in a patient
comprising the step of administering to the patient a
therapeutically effective amount of methioninase. In a
preferred aspect the methioninase administered to the patient is

30   substantially free of endotoxin. In another preferred aspect
the methioninase is conjugated to a polymer. In yet another
preferred aspect a method is provided at preventing
cardiovascular disease in a patient comprising the step of
administering to the patient a therapeutically effective amount

35   of a methioninase that is substantially free of endotoxin

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wherein the methioninase is also conjugated to polyethylene glycol. Example 7 provides one example of *in vivo* homocysteine depletion.

5   H.   Use of Methioninase for Tumor Imaging
            In another aspect of the invention a method is provided of
diagnosing a tumor in a patient comprising the steps of
depleting ^{12}C methionine in a patient by administering
methioninase to the patient followed by repleting the methionine
10   in the patient by administering ^{11}C methionine to the patient and
finally detecting the presence of ^{11}C methionine in the tumor
cells of the patient. The ^{11}C methylation may be detected by
means of, for example, but not limited to, positron emission
tomography (PET) scanning. Those of ordinary skill in the art
15   are familiar with other methods of detecting ^{11}C uptake by
cancerous cells. Such methods are described in, for example,
Lapela et al., J. Nucl. Med. 35:1618-23, 1994; Miyazawa et al.,
J. Nucl. Med. 34:1886-91, 1993; Leskinen-Kallio et al., J. Nucl.
Med. 33:691-95, 1992; Shields et al., J. Nucl. Med. 33:581-84,
20   1992; Huovinen et al., Brit J. Cancer 67:787-91, 1993; Dethy et
al., J. Nucl. Med. 35:1162-66, 1994; Lindholm et al., J. Nucl.
Med. 34:1711-16, 1993; Leskinen-Kallio et al., J. Nucl. Med.
32:1211-18, 1991; and Mineura et al., J. Nucl. Med. 32:726-28,
1991, all hereby incorporated by reference herein.
25   In a preferred aspect of the invention, the methioninase
provided is substantially free of endotoxin.
            In another preferred aspect of the invention the
methioninase was conjugated to a polymer, such as polyethylene
glycol.

30
                              Examples
            The following examples relating to this invention are
illustrative and should not, of course, be construed as
specifically limiting the invention. Moreover, such variations
35   of the invention, now known or later developed, which would be

within the purview of one skilled in the art are to be
considered to fall within the scope of the present invention
hereinafter claimed.

5   Example 1
                  Scaled Up Production of Methioninase
        A strain of Pseudomonas putida was modified and selected
for kanamycin resistance and for high levels of methioninase
expression as follows:
10   ATCC 8209 Pseudomonas putida and ATCC 77100 E. coli were
purchased from the ATCC in Oct. 1993. ATCC 77100 was grown in
LB (Sambrook et al., Molecular Cloning: A laboratory manual A.1,
1989) with 50 ug/ml kanamycin. Plasmid pCN 51, a shuttle vector
containing a kanamycin resistance gene that is able to replicate
15   in both P. putida and E. coli (Nieco et al., Gene 87:145-149,
1990, hereby incorporated by reference herein was isolated from
ATCC 77100 and purified using the Triton-lysozyme method
(Sambrook et al., Molecular Cloning: A laboratory manual 1.29-
1.30, 1989). ATCC 8209 was grown in LB medium and transformed
20   with pCN 51 using standard transformation procedures such as
those described in, for example, Sambrook et al., Molecular
Cloning: a laboratory manual. 1.74-1.84, 1989. The Kanamycin-
resistant strain was selected with kanamycin (100 ug/ml) in LB
medium and further grown in LB plates (Sambrook et al.,
25   Molecular Cloning: A laboratory manual A.1-A.4, 1989) in 100
ug/ml kanamycin. A single colony was grown in 100 ug/ml
kanamycin in LB overnight and then put under high-methioninase-
expression conditions: 10% LB, 0.1% potassium phosphate buffer
pH 7.2, 0.1% urea, 0.025% yeast extract, 0.01% magnesium sulfate
30   and 0.25% methionine with 50 ug/ml kanamycin for 24 hours. The
expression of methioninase was measured with the standard
methioninase assay as described herein. The methioninase over-
producing strain of Pseudomonas putida was selected and named as
AC-1.

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A scaled up fermentation process for AC-1 _Pseudomonas putida_ was then employed. An AC-1 single colony was grown in an LB plate containing 50 μg/ml kanamycin. The selected colony was incubated in 5 ml LB containing 50 μg/ml kanamycin at 26°C with

5   shaking at 250 rpm/min for 18 hours. Two ml of bacteria were amplified in 600 ml LB containing 50 μg/ml kanamycin at 26°C, with shaking at 200 rpm/min for 6 hours. Then 50 ml of bacteria were incubated in 2 liters LB containing 50 uM/ml kanamycin at 26°C with shaking at 100 rpm/min overnight (18 hours). Two

10   liters of bacteria (OD_{600}1.2-1.6) were then grown in a 40-liter tank in a special medium which contained 10% LB, 0.1% potassium phosphate buffer pH 7.2, 0.1% glycerol, 0.1% urea, 0.025% yeast extract, 0.01% magnesium sulfate and 0.25% methionine under high aeration conditions at 26°C for 24 hours. The OD_{600} reached 1.2-

15   1.8 and the activity reached 20-30 units/liter. The optimal cell density for harvesting is an OD_{600} of 1.5-1.8, with 2-3 mg/l of methioninase. This is equivalent to 1 kg of wet cells/400 liters. Preferably, a fully-equipped fermenter would be used with an approximate yield of 1 kg wet cells/10-20 liters. The

20   cells were harvested with an AGT column (Model UFP-500-E-55 car-tridge, A/G Technology Corporation) keeping the temperature at 4°C then centrifuged using an automatic refrigerated centrifuge (Sorvall superspeed RC2-B) at 4°C, at 9 krpm for 10 minutes. The cell pellet was then collected.

25   The cells were then suspended in extraction solution (20 mM potassium phosphate, pH 9.0) at a density of 500g wet cells/liter, and disrupted using three passages with a cavitator homogenizer (Microfluidics Corp. Model #HC 8000). The homogenate was stored at -800C immediately after cell breakage.

30   The specific activity of methioninase in the homogenate was 0.08-0.1 units/mg protein.

The AC-1 homogenate was suspended in extraction buffer (10 mM potassium phosphate pH 7.2, 10 uM pyridoxal phosphate, 0.01% beta-mercaptoethanol, 1 mM EDTA and 20% ethanol and was heated

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at 50°C for 2 minutes. The heating step may be performed for any
length of time sufficient to precipitate heat-sensitive
contaminating proteins while maintaining methioninase activity.
The suspension was centrifuged at 12 krpm at 4°C for 30 minutes.
5   The supernatant was collected and ultrafiltered with a Millipore
Prep/Scale- TFF PLHK 100k2.5 ft² cartridge. The pH was then
adjusted to 7.2.

A sample of about 25-35 g of total protein in 10 L
extraction buffer (10 mM potassium phosphate buffer pH 7.2 10 µM
10 pyridoxal phosphate and 0.01% J-mercaptoethanol) was applied to
a Toyopearl DEAE-650M column 10cm x 50cm (Toso Haas Japan) which
was pre-equilibrated with 10 mM potassium phosphate buffer (pH
7.2). The column was pre-washed with 20-30 L of 40 mM potassium
chloride in 10 mM potassium phosphate buffer, pH 7.2 containing
15 10 uM pyridoxal phosphate and 0.01% beta-mercaptoethanol, until
the OD$_{280}$ dropped below 0.1. The column was then eluted with a
linear gradient of potassium chloride at concentrations starting
at 40 mM, increasing up to 300 mM in extraction buffer. Elution
fractions of 400 ml were collected. The fractions containing
20 methioninase were determined by methioninase activity assay as
described herein and were pooled. The pooled fractions were
pre-equilibrated with 10 mM potassium phosphate buffer, pH 8.3,
containing the same concentration of pyridoxal phosphate and
beta-mercaptoethanol, and 150 mM potassium chloride.

25 A sample of about 1-2 g of total protein obtained from the
methioninase peak of the DEAE-650M (5cm X 20cm) column in buffer
containing 10 mM potassium phosphate pH 8.3, 150 mM potassium
chloride, 10 µM pyridoxal phosphate and 0.01% J-mercaptoethanol
was applied to a DEAE-Sephadex A50-column. The column was
30 eluted with a 150-500 mM KCl linear gradient in 10 mM potassium
phosphate buffer, pH 8.3 containing 10 µM pyridoxal phosphate and
0.01% beta-mercaptoethanol. Elution fractions of 150 ml were
collected. The fractions containing methioninase determined by
activity assay as described herein were pooled. The pooled
35 methioninase was then further purified to remove endotoxin. The

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sample was pre-equilibrated by dialysis in 0.12M sodium chloride
and 10 mM sodium phosphate buffer, pH 7.1. The sample was
applied to a 5cm X 15 cm Acticlean Etox resin column (Sterogen
Bioseparations, Arcadia, CA). The enzyme was then eluted from
5   the column using the same buffer, eluant fractions containing
methioninase activity were collected, and the amount of
endotoxin was then determined on the eluted fractions. Table 1
shows the results of this purification method.

Table 1					
Scale Up Of Methioninase Manufacturing Process					
Step	Volume (ml)	Activity Units/ml Total	Protein mg/ml (g) Total	Specific Activity (units/mg)	Yield PH (%)
Homogenate	3850	1.05/5200	11.5/44.3	0.1/6.7	100
Heat	3130	1.43/4472	4.4/13.8	0.33/6.9	86
Ultrafiltration	2950	1.41/4160	4.25/12.5	0.33/7.2	80
First Column	850	4.42/3744	1.64/1.39	2.6/7.2	72
Second Column	1000	3.22/3220	0.22/0.22	14.6/8.3	62
Third Column	540	4.8/2620	0.34/0.18	14.3/7.1	52
Concentration	16.5	153/2529	10.5/0.173	14.8/7.1	48

10

An analysis of the purified methioninase on a 7.5% SDS-PAGE
gel showed a single band of protein of 43 kd, representing one
subunit of the 172 kd protein was observed when the active
15   fractions from the second column were analyzed by SDS-PAGE. The
purity of the methioninase isolated by this method was 98.7% as
seen using HPLC analysis, which demonstrated only one peak of
protein.

The extraction methods described herein resulted in a
20   preparation of substantially isolated methioninase that was
substantially free of endotoxin. Those of ordinary skill in the
art will recognize that modifications may be made to the
extraction methods described in and are included within the
scope of the invention.

25

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Example 2

Dependence of Human Tumors on Elevated Levels of Methionine

Methionine dependence occurs frequently in human cancer.
Twenty NCI human tumor cell lines from all major organ systems

5   were tested for methionine dependence. All twenty cell lines
were found to be methionine dependent. In addition, fresh human
tumor specimens were tested and found to be methionine
dependent. Normal human cell lines were not methionine
dependent.

10   Figure 1 shows the results of one such test. In Figure 1,
normal cell strains and tumor cell lines were grown in Eagle's
MEM with 10% dialyzed serum that was either: methionine-
containing homocysteine-free (MET$^+$HCY$^-$); methionine-free,
homocysteine-containing (MET$^-$HCY$^+$) or methionine-free,

15   homocysteine-free (MET$^-$HCY$^-$). Kidney, colon, lung and prostate
cancer cell lines and melanoma and normal fibroblast strains
were screened in methionine (MET)-containing medium, MET-free
medium, homocysteine (HCY)-containing medium and in MET-free
medium, homocysteine (HCY)-containing medium and in MET-free,

20   HCY -free medium. The cells were grown in Eagle's Minimum
Essential medium (MEM medium) supplemented with 10% dialyzed
fetal bovine serum, 10 μM hydroxocobalamin and 100 uM folic acid.
The medium was supplemented with or without methionine in the
following way: MET+HCY: The medium was supplemented with 100 μM

25   L-methionine without homocysteine. METHCY+: The medium was
supplemented with 200 μM D,L-homocysteine without methionine.
METHCY: The medium was methionine-free and homocysteine-free.
The fetal bovine serum was dialyzed against phosphate buffered
saline (PBS) (serum:PBS of 1:12) 10 times.

30   Cell proliferation was determined by metabolic reduction of
the dye XTT as measured by absorption at 450 nm. Cells were
grown on 48-well dishes by inoculation of between 5 X 10^3 and 2 X
10^4 cells per well. XTT (2,3-bis(2-methoxy-4-Nitro-5-
sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium

35   hydroxide), which is metabolically reduced in viable cells to a

water soluble formazan product was used to determine cell
proliferation. The absorbance of the reduced product was
measured at 450 nm. XTT was prepared at 1 mg/ml in pre-warmed
(37°C) PBS, Phenazine methosulfate (PMS) was prepared at 5 mM in
5   PBS. Fresh XTT solution was mixed with PMS stock solution at a
.   ratio of 5 11 PMS per 1 ml XTT. Each well was filled with 1 ml
medium. 0.25 mL of mixed XTT were added to each well. The
absorbance of reduced XTT was read at 450 nm with a Hitachi U-
2000 spectrophotometer after 3 hours of incubation. The OD of
10   XTT was measured every 3 days after the cells were cultured for
an initial 24 hours. Foreskin fibroblast strains FS-3 and HS-68
proliferated essentially equally in MET^+HCY^- or MET^-HCY^+.
However, a new observation is reported here in that both
fibroblast strains could proliferate to some degree and then
15   maintain themselves for at least 16 days in MET HCY medium.

     Table 2 illustrates the results of a cell proliferation
assay of 20 tumor cell lines. In the assay, $5 \times 10^3 - 2 \times 10^4$
cells/well of cell suspension were cultured in 48-well plates in
MEM with 10% fetal bovine serum for 24 hours. Cells were washed
20   with Hanks basic salt solution (HBSS) twice and then were
divided into 3 groups. Group I: Cells cultured in MET^+HCY^-
medium; Group II: Cells cultured in MET^-HCY^+ medium; Group III:
Cells cultured in MET^-HCY^- medium. Cells were cultured in a
gassed incubator with 95% air/5%CO_2. The media were changed
25   every 2-3 days. In Table 2, strong growth of the cell lines is
indicated with a +++, some growth with a ++, slight growth with
a + and no growth with a -. Approximately one-half of the tumor
cell lines could not grow in the MET-free HCY-containing medium
with the rest growing to varying degrees whereas the same medium
30   allowed the normal cells to grow as well as in MET-containing
medium. Although the normal cell strains could grow to some
extent and then maintain themselves for two weeks or more in
MET-free, HCY-free medium, all 20 tumor cell lines tested died
in this medium suggesting that potentially all cancers can be
35   selectively killed by MET deprivation. This example provides

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evidence that methionine dependence may be a universal and
selective target for cancer therapy, especially with
methioninase as the therapeutic.

5

Table 2				
Cell Lines	Type of Cancer Cell	Proliferation		
		MET^+HCY^-	MET^-HCY^-	MET^-HCY^-
Tumor				
A498	Renal	+ + +	-	-
CAKI	Renal	+ + +	+ +	-
SN12C	Renal	+ + +	+	-
RXF-393	Renal	+ +	+	-
DU-145-1	Prostate	+ + +	-	-
PC-3	Prostate	+ + +	-	-
H322M	Lung	+ + +	-	-
H23	Lung	+ + +	+ + +	-
SNB-75	CNS	+ + +	+ +	-
SF-295	CNS	+ + +	+ +	-
H460	CNS	+ + +	-	-
H522	CNS	+ + +	+ +	-
HCT-15	colon	+ + +	-	-
Colo 205	Colon	+ + +	+ + +	-
SW-620	Colon	+ + +	-	-
HT29	Colon	+ +	+	-
UACC-257	Melanoma	+ + +	+ + +	-
UACC-62	Melanoma	+ + +	+ + +	-
SK-MEL-5	Melanoma	+ +	+	-
Lox	Melanoma	+ +	-	-
Normal Cells				
FS-3				
HS-68	Foreskin	+ + +	+ + +	+
	Foreskin	+ + +	+ + +	+

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Example 3
Inhibition of Human Tumor Growth by Methioninase in Mouse
Xenograft Models

To test the efficacy of methioninase on the reduction of

5   tumor growth, a mouse xenograft model was used. A human lung
cancer tumor (H460) was transplanted in the subcutis of nude
mice. The mice were divided into four groups of four mice each.
Four days after the transplantation, group A was administered
0.4 ml buffer (0.12 M sodium chloride, 10 mM sodium phosphate pH

10   7.1) by intraperitoneal injection every 4 hours; group B was
administered methioninase (1 unit/g) by intraperitoneal
injection every 4 hours; group C was administered 5-FU (60mg/kg)
by intraperitoneal injection every 4 days; and group D was
administered with vincristine (VCR) (0.9mg/kg) by

15   intraperitoneal injection every 4 days. Tumor size and body
weight were measured every two days. The results are shown in
Figures 2 and 3. The results indicate that treatment with
methioninase greatly slowed the growth of the H460 human non-
small-cell-lung cancer in nude mice. In these experiments, the

20   mice were fed normal, not methionine-depleted, diets. The
efficacy of methioninase against H460 was in stark contrast to
5-fluorouracil and vincristine treatment, which were inactive
against this tumor. The activity of the administered
methioninase did not cause weight loss for up to 10 days

25   treatment at 40-120 units/day indicating the possibility of low
toxicity. In contrast, vincristine caused weight loss,
indicating toxicity. Thus, methioninase is a highly effective
antitumor agent with a new tumor-selective mode of action with
minimal toxicity, demonstrating potential clinical effectiveness

30   against human solid tumors.

Example 4
Preparation of PEG-methioninase

Methoxy polyethylene glycol succinimidyl carbonate, (M-SC

35   5000 PEG), molecular weight 5000, was dissolved in 20 mM sodium

phosphate buffer (pH 8.3) at a concentration between 2 mM and
20 mM. The molar ratios of M - SC 5000 PEG to methioninase were
varied from 6:1 to 240:1. The PEGylation reactions were carried
out in reaction buffer (25 mM sodium phosphate buffer, pH 8.3),
5   at 4° C or 20° C from 30 minutes to 60 minutes. The reactions were
stopped with stop buffer (0.14 M sodium phosphate buffer, pH
6.5) at 0°C. Unreacted M - SC 5000 PEG was then removed by gel
filtration chromatography as described herein. The resulting
PEG - methioninase was formulated in 0.12 M sodium chloride and
10   10 mM sodium phosphate (pH 7.2) with the use of 30k Amicon
Centriprep concentrators. The PEG-methioninase was then
sterilized by filtration with a 0.2 uM micron membrane filter.
The PEG-methioninase may be stored at -70° C for up to 6 months
without loss of activity.

15   Methioninase activity was measured. The activity of PEG-
methioninase was maintained to at least 60% of the activity of
nonPEGylated methioninase. PEG-methioninase was analyzed by
both native and SDS polyacrylamide gel electrophoresis as
described by Laemmli and by Sambrook (Laemmli, Nature 227-680,
20   1970; Sambrook et al., Molecular Cloning: a laboratory manual
18.47-18.59, 1989) and modified as follows: Electrophoresis of
PEG-methioninase was carried out in 7.5% polyacrylamide-
precasted gels in 0.2 M Tris-glycine buffer (pH 8.3), with or
without SDS. The gels were stained with Coomassie blue and
25   destained with 40% methanol 10% acetic acid. No unmodified
methioninase band was seen when PEG-methioninase was applied to
native gels, although a very small band could be seen in SDS
gels.

PEG-methioninase was then analyzed by an HPLC gel
30   filtration column as follows: PEG-methioninase (20 1l at 1-2
mg/ml) was applied using a 20 μm loop to a Progel-TSK G3000 SW
(Supelco) column in 0.2M sodium phosphate buffer (pH 7.2) and
eluted with 0.2M sodium phosphate buffer (pH 7.2) at a flow rate
of 1.0 ml/mn. Only one peak of protein was detected, the PEG-
35   methioninase peak, which was free of unreacted PEG; no non-

PEGylated methioninase peak could be detected. The purity of
PEG-methioninase was approximately 100%.

    a)   Time Course Studies of the PEGylation Reaction
5   The time course of the conjugation of polyethylene glycol
to methioninase was measured as follows: 0.07 mM of methioninase
was reacted with 0.4 mM of M - SC 5000 PEG in 0.2 M sodium
phosphate buffer (pH 8.3) for various time periods up to 2
hours at 4°C. The reactions are stopped at various time points
10   with 0.2 M sodium phosphate pH 6.5). Non - reacted PEG was
removed with a 30k Amicon centriprep concentrator. The results
are shown in Table 3.
        PEG-methioninase was analyzed on native and SDS-PAGE gels
as described herein. The appearance of a higher molecular
15   weight band was measured and is indicated with -, +-, +, or ++
symbol in Table 3, with - indicating the lack of PEG-
methioninase or methioninase, +- indicating small amounts of
PEG-methioninase or methioninase, and ++ indicating large
amounts of PEG-methioninase or methioninase. Table 3 also
20   indicates the activity of the PEG-methioninase or methioninase.

Table 3 Time Course of Methioninase Pegylation									
	Molar Ratio (M*:P**)	Condition		Time Course (Min)					
		°C	pH	0	4	30	60	90	120
Activity(%)	1:6	4	8.3	100	99	77	70	60	58
Native PAGE	Methioninase			++	+	+-	-	-	-
	PEG-methioninase			-	+	+	++	++	++
SDS PAGE	Methioninase			++	++	++	+	+	+-
	PEG-methioninase			-	+-	+	+	++	++

*M=methioninase   **P=M-SC 5000-PEG.

25   b)   Concentration dependence of Methioninase PEGylation
              with the M-SC 5000 PEG
        0.07 mM of methioninase was reacted with various
concentrations (0.4 mM to 4.0 mM) of M - SC 5000 PEG in 0.2 M
sodium phosphate buffer (pH 8.3). The molar ratios were 1:6 -

1:60.   The reactions were carried out at the same conditions as
in Example a for 60 minutes at 4°C.

The results are shown in Table 4.

Table 4 Concentration Dependence of Methioninase Pegylation								
	Condition		Molar Ratio (P**:M*)					
	°C	min	0	6	12	24	48	60
Activity (%)	4	60	100	79	69	48	33	31
Native-PAGE	M*		++	-	-	-	-	-
	P-M***			-	++	++	++	+++
SDS-PAGE	M*		++	+	+	+-	+-	+-
	P-M***		-	+	++	++	++	+++

*M: methoninase. **P: PEG. ***P-M: PEG-methioninase.

c)   Pharmokinetics of PEG-Methioninase

Molar ratios of PEGylation of methioninase with M – SC 5000
PEG to methioninase were 1:6 (P1) and 1 : 12 (P2)
respectively. The reactions were carried out at the same
conditions as in (b) above. The purified P1 and P2 as well as
unmodified methioninase were injected into the tail vein of
three different groups of mice, respectively. The blood samples
were collected at 0, 1, 2, 3, 4 and 20 hours. The activity
assay of methioninase and PEG – methioninase and the level of
depletion of methionine were measured. The results are shown in
Table 5.

Table 5 Pharmacokinetics of PEG-Methioninase								
Mouse Group		Treatment Agent Condition Dost Route	Methioninase Activity in Sera Half-life (Relative % hours)					
			0	1	2	3	4	20 (hours)
I	M*	Buffer 8 units i.v. 0	100	49	32	8	2	1
II	P1**	M:P = 1:6 8 units i.v. 0	100	66	56	41	8	3.3
III	P2**	M:P = 1:12 8 units i.v. 0	100	94	72	56	18	4.4

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*M: methioninase.   **P, P1, P2:   PEG-methioninase

Example 5

Antigenicity experiment using PEG-methioninase in Guinea Pigs:

Methioninase was reacted with M – SC 5000 PEG at molar ratios of 1:60, 1:120 and 1:240, respectively, at 20^0 C for 30 minutes. The other conditions are the same as in (a) above. PEG-methioninase was analyzed by the methioninase activity assay, electrophoresis and HPLC. No band or peak of unmodified methioninase was detectable. Thus, PEG-methioninase was the only source of enzyme.

2 mg of methioninase or PEG-methioninase was administered intraperitoneally to guinea pigs every two days for three times total. Two weeks later, 4 mg of methioninase or PEG-methioninase was administered intravenously. 0.2 sodium phosphate buffer and BSA were used as negative and positive controls, respectively, in this experiment. The results are evaluated according to the criteria in Table 6. The results are shown in Table 7. PEGylation of methioninase results in a composition of extremely low antigenicity as tested in guinea pigs. Guinea pigs are known to be more sensitive than humans to antigens.

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TABLE 6

EVALUATION CRITERIA OF PEG-METHIONINASE ANTIGENICITY STUDY IN
GUINEA PIGS

5
0. Normal	1. Restlessness
2. Piloerection	3. Trembling
4. Running Nose	5. Sneezing
6. Coughing	7. Hyperpnea
8. Urination	9. Evacuation
10. Lacrimation	11. Dyspnea
12. Rhonchus	13. Cyanosis
14. Staggering gait	15. Jumping
16. Gasping & writhing	17. Convulsion
18. Side position	19. Cheyne-stokes respiration
20. Death	

EVALUATION CRITERIA

(-)	negative:	no change
(+)	slight:	1 - 4
(++)	moderate:	1 - 10
(+++)	severe:	1 - 19
(++++)	death:	20

Table 7 Antigenicity of PEG-ONCASE in Guinea Pigs					
Sensitization			Induction		Evaluatio
(Inject every two days, 3 times)		(Two weeks later)	WHO Criteria		
	Antigen	Doses	Antigen	Doses	
A	Buffer	0.2 ml, ip	Buffer	0.2 ml, ip	-
B	BSA	1.2 ml, ip	BSA	10 mg, iv	++++
C	Methioninase	2.0 mg, ip	Methioninase	4.0 mg, iv	++++
D	PEG-Methioninase	2.0 mg, ip	PEG-Methioninase	4.0 mg, iv	-

25   The results showed that PEG-methioninase, in contrast to
Bovine Serum Albumin (B.S.A.) and methioninase, has no

antigenicity in guinea pigs, which suggests that PEG-
methioninase is non-immunogeneic when administered to a mammal.

Example 6

5   Administration of Methioninase for Human Cancer Therapy

A Phase I dose-escalating study of methioninase has been
started to determine methioninase toxicity, pharmacokinetics and
maximum tolerated dose. A 2-hour infusion of 5,000 units (0.5g)
and 10,000 units (1.0g) of methioninase has been administered by
10   iv infusion for 2 hours into 2 patients with advanced breast
cancer. Blood and urine samples were obtained at frequent
intervals from 0 to 24 hours. The toxicity evaluations were
carried out according to the WHO grading system.
Pharmacokinetic data were obtained for both methioninase and
15   methionine levels in the serum.

No acute clinical toxicity was observed whatsoever with all
the toxicity criteria measured having a minimum grade of 0. The
half-life of methioninase was 2 hours and 3.2 h, respectively,
in patient 1 and patient 2. The depletion of serum methionine
20   started within 30 minutes of the infusion, and was maintained
for 4 hours after the infusion was completed. The lowest serum
methionine levels were 35% and 19% of the pretreatment level,
respectively, in patient 1 and patient 2. The results are shown
in Tables 8-10 and Figures 4-6. The patients were treated as
25   follows:

Patient 1: Date: December 14, 1994. Diagnosis: Breast
cancer with axillary lymph node and lung metastasis. Female, 46
years old. 5,000 units (0.4 g) of methioninase purified
30   according to extraction method (Example 1) was administered in a
200 ml intravenous infusion in 2 hours. Blood samples were
collected before the treatment and after every two hours up to
20 hours. The levels of methionine and methioninase were
measured and the relative levels calculated, Figure 4.

35

Patient 2: Date: February 2, 1995. Diagnosis: Breast
cancer with axillary lymph node metastasis. Female, 54 years
old. 10,000 units (0.8g) of methoninase purified according to
Extraction Method (Example 1) was administered in a 400 ml

5   intravenous infusion in 2 hours. Blood samples were collected
before the treatment and after every two hours up to 20 hours.
The levels of methionine and methioninase were measured and the
relative levels calculated, Figure 5.

10   Patient 3: Date: October 15, 1995. Diagnosis: Breast
cancer with axillary lymph node metastasis. Female. 45 years
old. 20,00 units (1.6 grams) of methioninase purified according
to Extraction Method (Example 1) was administered in a 1000 ml
intravenous infusion in 10 hours. Blood samples were collected

15   before the treatment and after every two hours up to 20 hours.
The levels of methionine and methioninase were measured and the
relative levels calculated, Figure 6.

Patient 3 maintained serum levels of methioninase as high

20   as 50% of the maximum level for a subsequent 6 hours after
infusion. Methionine depleted over 200-fold, from 23.1 uM to
0.1 uM, by the tenth hour of infusion. No clinical toxicity was
observed whatsoever in all the toxicity criteria mearsued.

25   Toxicity evaluation was performed according to WHO toxicity
criteria in four areas: a. History and diagnosis data; b.
Physical examination; c. Laboratory evaluation; and d.
Pharmacokinetics evaluation (methioninase and methionine levels
in serum). The results, shown in Tables 8-11, and Figures 4-6,

30   demonstrate that the methioninase of the present invention is
not toxic and may be used to decrease methionine levels in
humans.

Table 8 Clinical Phase I Trial for Methioninase			
	Patient I	Patient II	Patient III
Diagnosis	Breast cancer with metastasis in axillary lymph node. Patient 1 had lung metastasis as well		Breast cancer/metastasis
Sex	Female	Female	Female
Age	46	54	45
METase	5,000 units	10,000 units	20,000 units
I.v. infusion	2 hours	2 hours	10 hours
Blood collection	Before infusion and during infusion every two hours, after infusion 10 hours		
Evaluation	WHO criteria		

Table 9 Toxicity of Clinical Phase I Trial for Methioninase			
Physical & Laboratory Examination	Toxicity Grade of FDA Standard		
	Patient I	Patient II	Patient II
Hematological	0	0	0
Gastrointestinal	0	0	0
Renal	0	0	0
Pulmonary	0	0	0
Fever	0	0	0
Allergic	0	0	0
Phlebitis	0	0	0
Cutaneous	0	0	0
Cardiac	0	0	0
Neurological	0	0	0

Table 10 Pilot Phase I Clinical Trial Methionine and Homocysteine Levels in Patient III (Methioninase 20,000 units iv infusion for 10 hours)					
Time		Homocysteine		Methionine	
		μM	%	μM	%
Before infusion		7.7	100	23.1	100
During infusion	2h	5.4	70	2.9	12.6
	4h	5.6	73	3.4	14.7
	6h	4.8	62	0.5	2.2
	8h	4.2	55	0.2	0.09
	10h	2.8	36	0.1	0.04
After infusion	2h	2.6	34	0.2	0.01
	10h	2.5	32	0.7	0.3

5

Table 1♦ VITAL PARAMETERS OF PATIENTS TREATED WITH METHIONINASE

Patient	Treatment	Temperature	Pulse	Respiration Rate	Blood Pressure
#1 O.R.L.	Before	36.7	134	21	110/70
	During (Max)	36.8	138	22	120/80
	(Min)	36.4	125	24	110/70
	After	36.6	127	24	120/80
#2 S.N.K.	Before	36.7	74	14	118/78
	During (Max)	36.6	74	12	120/80
	(Min)	36.6	70	14	110/70
	After	36.7	74	14	110/74
#3 S.N.O.	Before	36.4	67	14	124/82
	During (Max)	37.0	76	14	128/74
	(Min)	36.0	66	12	90/70
	After	36.3	65	12	114/88

Example 7

In vivo Depletion of Homocysteine

The *in vivo* effectiveness of methioninase for use in
depleting homocysteine according to the present invention was
demonstrated by administration of the enzyme to mice as follows:
4 units of methioninase purified according to the Method of
Example 1 was injected intraperitoneally in mice. About one
hour after the injection, the blood concentrations of
methionine, homocysteine, and cysteine were assayed by
subjecting 50 μl of mouse serum after derivitization with PITC,
to analysis by reverse phase FPLC. The chromatographic profile
was compared to that of PITC derivatized internal methionine,
homocysteine, and cysteine standards. The sensitivity of the
assay was about 0.5 μM methionine.

As shown in Table 12, compared to the levels of
homocysteine and cysteine in control, untreated mice,
methioninase had significant *in vivo* homocysteine depletion
activity without any significant depletion of *in vivo* levels of
cysteine.

Table 12 *In Vivo* Reduction of Methionine and Effects on Other Amino Acids by Methioninase			
Sample	Methionine (μM)	Homocysteine (μM)	Cysteine (μM)
Control	131.8	2.7	74
Methioninase	6.6	0.5	71

Example 8

Use of Methioninase for Tumor Imaging

[^{12}C] methionine is depleted in a patient by the
administration of purified methioninase as described herein.
Preferably the methioninase is free of endotoxin. Methioninase
(10,000-20,000 units) is administered by intravenous infusion
over 4-8 hours. Approximately 5-50 mCi of [^{11}C] methioninase is
then administered. Positron emission tomography (PET) imaging

is then used to detect the uptake of the [^{11}C] tracer by the
patient's cells. Tracer uptake is quantitated by calculating
both the standardized uptake value (S.U.V.) and the kinetic
influx constant (Ki) value by means known to those of ordinary
5   skill in the art. Elevated levels of [^{11}C] methionine in cells
indicates that such cells are likely to be tumor cells. The
preferential [^{11}C] methionine uptake by the tumor cells provides
a means of selectively detecting tumor cells among normal cells.

        The foregoing specification, including the specific
10   embodiments and examples, is intended to be illustrative of the
present invention and is not to be taken as limiting. Numerous
other variations and modifications can be effected without
departing from the true spirit and scope of the present
invention.

15

                         Example 9
        Isolation of Nucleic Acid Molecules Encoding Methioninase
PCR Reaction of the Insert of Methioninase Gene Clone:
        Genomic DNA of *Pseudomonas putida* AC-1, derived from
20   ATCC8209, was used as template; the primers used were as
follows:

        t1:5'-GCCGGTCTGTGGAAT*AAGCT*-3' (Sense),
                        HindIII
25
        t2:5'-CCAG*GGTCGA*CTCCAGCGCC-3' (Antisense).
                  Sal I

        The PCR reaction condition was as follows: first denaturation
30   at 95°C for 10 minutes, then 5 cycles of denaturation at 94°C
for 30 seconds, annealing at 60°C for 30 seconds, and
extension at 72°C for 2 minutes; then 25 cycles of
denaturation at 94°C for 30 seconds, 60°C for 30 seconds, then
extension at 72°C for 1.5 minutes; then final extension at
35   72°C for 10 minutes. The PCR amplified products are two bands
of which the 1365 bp band was collected, and purified as the
insert ONCase-1 DNA.

Cloning and Transformation

        The ONCase-1 DNA was ligated with pT7Blue T-vector

(Novagen) at the EcoR V T-cloning site. The pONCase-1 DNA was

5   transformed into DH5-α bacterial cells using standard

procedures.

DNA Sequencing

        DNA sequencing was performed using T7 DNA polymerase and

10   the dideoxy nucleotide termination reaction. The primer

walking method was used. [^{35}S] dATP was used for labeling.

Sequencing reactions were analyzed on 6% polyacrylamide wedge

or non-wedge gels containing 8M urea. DNA samples were loaded

in the order of ACGT. DNA sequences were analyzed by

15   MacVector. The DNA sequence and corresponding amino acid

sequence are provided in Figure 8.

Example 10

High Expression Clones of Recombinant Methioninase

20   PCR Reaction of the Insert for the Methioninase Expression

Clone:

        The pONCase-1 clone was used as the template, the primers

used are as follows:

25   t14. 5' - GGAATTC*CATATG*CACGGCTCCAACAAGC - 3' (Sense)
                         NdeI

t15. 5' - AGTCAT*CCTAGG*TCACATCATCATCATCATCATGGCACTCGCCTTGAGTGC-

30   3'
                  BamHI   (Antisense)

t18. 5' - AGTCAT*CCTAGG*TCAGGCACTCGCCTTGAGTGC - 3' (Antisense)

35   　 BamHI

The PCR reaction condition was as follows: first denaturation

at 95°C for 10 minutes, then 5 cycles of denaturation at 94°C

40   for 1 minute, annealing at 56°C for 1.5 minutes, and extension

at 72°C for 2 minutes; then 20 cycles of denaturation at 94°C
for 30 seconds, 56°C for 30 seconds, then extension at 72°C
for 1.5 minutes; then final extension at 72°C for 10 minutes.
Two PCR amplified products, ONCase-2 (1238bp), ONCase-3
5   (1220bp) band were collected and purified.

Cloning and Transformation
        The DNA of ONCase-2 and ONCase-3 DNA was digested with
NdeI and BamHI and ligated with the pT7.7 vector at the NdeI
10 and BamHI cloning sites. The pONCase-2 and pONCase-3 DNA
sequences were then transformed into BL21 (DE3) bacterial
cells using standard procedures.

Selection of pAC-1 and pAC-2 Clones
15   The positive clones were selected from Ampicillin-
containing plates. After storage at 4°C for 24 hours, the
positive clones which expressed high level of recombinant
methioninase had a distinct pink color that allowed their
identification and selection. The methioninase expression
20 levels of the positive clones were determined by activity
assay. Two high expression clones were selected as the pAC-1
clone which contained ONCase-3 and as the pAC-2 clone which
contained ONCase-2.

25 Construction of pAC-3 Clone and pAC-4 Clone
        The tetracycline resistance gene was obtained from pBR322
at the Ava I and Cla I sites. The Ava I end was filled into a
blunt end, and was ligated with pAC-1 which was digested with
the BamH I and Cla I restriction enzymes, with the BamH I end
30 filled into a blunt end. Positive clones which became pink
after storage at 4°C for 24 hours were selected from
Tetracycline-containing plates. A high expression recombinant
methioninase clone was determined by activity assay and named
as the pAC-3 clone.

The Tetracycline-resistance gene was also obtained from pBR322 at the Ava I and Hind III sites. The Ava I end was filled into a blunt end, and was ligated with pAC-1 which was digested with the Hind III and Cla I restriction enzymes, with the Cla I end filled into a blunt end. Positive clones which became pink after storage at 4°C for 24 hours were selected from Tetracycline-containing plates. A high expression recombinant methioninase clone was determined by activity assay and named as the pAC-4 clone. A variety of high level expression clones are provided in Table 13 and Figure 9.

Table 13 rMETase Expression Clones					
Clone	Vector	Antibiotic Resistance	Promoter	Fusion	Expression* (g/1)
pAC-1	pT7.7	Amp	T7	---	1.0
pAC-2	pT7.7	Amp	T7	His. Tag	0.5
pAC-3	pT7.7	Tc	T7	---	0.5
pAC-4	pT7.7	Tc	T7	---	1.0
*Expression level in shaking flask (TB medium, 37°C, 400 rpm, 36 hours).					

Example 11

Fermentation of Recombinant Methioninase Expression Clones

The expression clones of recombinant methioninase were grown in Terrific Broth medium containing either Ampicillin (100μg/ml) or Tetracycline (10μg), at 28°C or 37°C with 400 rpm shaking in a 6-L flask or fermenter.

Example 12

Purification of Recombinant Methioninase

An outline of the purification method is provided in Figures 10 and 11.

(1) Pre-column treatment of the sample

The bacteria were harvested by centrifugation at 800 x g at 4°C for 10 min. The bacterial pellet is then suspended in extraction solution (20mM potassium phosphate pH9.0, 10 μM

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pyridoxal phosphate and 0.01% β- mercaptoethanol) and
disrupted with a cavitator -type homogenizer (Microfluidics
Corp. model # HC 8000). Heat treatment of the homogenate is
then carried out at 50°C for one minute. The suspension is
5   centrifuged with an automatic refrigerated centrifuge (SORVALL
Superspeed RC 2-B) at 4°C at 13 k rpm for 30 min. The
supernatant is then collected. This step is followed by
ultrafiltration by a Millipore Prep / Scale - TFF PLHK 100k
2.5 ft^2 cartridge with buffer (10mM potassium phosphate pH8.3).
10   The pH is adjusted to 7.2 by ultrafiltration.

(2)   Chromatographic conditions
The first column: DEAE Sepharose FF
Column: XK 100/60, Height: 32 cm, Volume: 2.5 L
15   Solution: [A] 40mM potassium chloride, 10mM potassium
phosphate (pH7.2) containing 10 μM pyridoxal phosphate
and 0.01% β - mercaptoethanol.
[B] 200mM potassium chloride, 10mM potassium phosphate
(pH7.2) containing 10 μM pyridoxal phosphate and 0.01% β
20   - mercaptoethanol.
Flow Rate: 5 ml/min.
Sample: About 100-200 g of total protein (10-20 mg/ml) are
applied on the first column.
Gradient: [1] Pre-wash with solution A approximately 10
25   volumes until the OD$_{280}$ drops below 0.1.
[2] Gradient: Solution B from 20% - 100%.
Fractions: Elution fractions of 200 ml are collected. The
fractions containing rMETase are identified by activity
assay and pooled.
30

The second column: DEAE Sepharose FF
Column: XK 50/30, Height: 25 cm, Volume: 500 ml
Solution: [A] 100mM potassium chloride, 10mM potassium
phosphate (pH8.3) containing 10μM pyridoxal phosphate and
35   0.01% β - mercaptoethanol.

[B]   200mM potassium chloride, 10mM potassium phosphate
(pH8.3) containing 10μM pyridoxal phosphate and 0.01% β −
mercaptoethanol.

Flow Rate: 5 ml/min.

5 Sample: Approximately 10-20 g of total protein (2-4 mg/ml),
after dialysis in 100mM potassium chloride, 10mM
potassium phosphate (pH8.3) containing 10μM pyridoxal
phosphate for 24 hours, are applied on the second column.

Gradient: [1]   Pre-wash with solution A approximately 5
10   volumes until the OD_{280} drops below 0.05.

[2]   Gradient: Solution B from 0% − 60%.

Fractions: Elution fractions of 200 ml are collected. The
fractions containing rMETase are identified by the
activity assay and pooled.

15

The third column: Sephacryl S-200 HR

Column: HiPrep 26/60, volume 320 ml.

Solution: 0.15 M sodium chloride in 10mM sodium phosphate
(pH7.2)

20 Flow Rate: 1.2 ml/ min.

Sample: Approximately 10 ml concentrated sample. (after
dialysis in 0.15 M sodium chloride, 10mM sodium phosphate
(pH7.2) for 12 hours), are applied to the third column.

Fractions: Elution fractions of 20 ml containing rMETase,
25   which are identified by yellow color and activity assay,
are collected.

The fourth column: Acticlean® Etox

Purified rMETase (10-20 mg protein / ml) in a volume of
30 100-200 ml is applied on a 500 ml Acticlean® Etox column, and
eluted with elution buffer (0.15 M sodium chloride in 10mM
sodium phosphate pH7.2) in order to eliminate endotoxin.
Acticlean® Etox is reusable and can be cleaned with 1 M sodium
hydroxide and can be autoclaved.

35

Concentration of the final eluant

The final eluant is concentrate with 30 K Amicon
Centriprep Concentrators. The formulation for purified
rMETase is 0.15 M sodium chloride, 10mM sodium phosphate,
5   pH7.2.

Purification of rMETase.Histidine: Chromatography on Ni^{++} Sepharose column

The cell homogenate, after pre-column treatment, is
10 suspended in binding buffer (5mM imidazole, 0.5 M NaCl, 20mM
Tris.HCL, pH7.9). The column is then washed with 10 volumes
of binding buffer followed by washes with 6 volumes of wash
buffer (60mM imidazole, 0.5 M sodium chloride, 20mM Tris, HCl,
pH7.9). Elution occurs after 6 volumes of elution buffer (1 M
15 imidazole, 0.5 M NaCl, 20mM Tris. HCl pH7.9) have been run
through the column. The fractions containing rMETase,
identified by yellow color, are collected.

Example 13
20   ## Analysis for The Purity of rMETase with HPLC
Column: SUPELCO, 8-08541, Progel TM - TSK, G 3000- SWXL, 30
cm x 7.8 mm.
Eluent Solution: 0.15 M sodium chloride in 10mM sodium
phosphate buffer (pH7.2).
25 Flow Rate: 0.1 ml/min.
Sample: 20 µl (0.1 - 1 mg/ml).

An example for production of rMETase is shown in Figures 10
and 11. Purity is shown in Figure 12.

30

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Example 14
Formulations Containing Recombinant Methioninase, Crystallized and Lyophilized Forms

Solution formulation:

5   rMETase is formulated in solution, 0.15M sodium
   chloride, 10mM sodium phosphate buffer (pH 7.2), at the
   concentration 10-20mg/ml. The stability of rMETase is
   showed in Figure 13.

10 Crystallized form:
      rMETase (10-20mg/ml), in a 0.15 M sodium chloride and 10
   mM sodium phosphate buffer (pH 7.2) was desalted using a
   Sephadex G-25 (DNA grade, superfine, Sigma) column. The
   solution was frozen on a dry ice and acetone bath and then
15 crystallized in a vacuum of 100 milifar, at -80°C, for 72 hours
   using a Verdis Freeze Mobil 24.

   Lyophilized form:
      rMETase (10-20mg/ml), in a 0.15 M sodium chloride and 10
20 mM sodium phosphate buffer (pH 7.2), was frozen on a dry ice
   and acetone bath and lyophilized in a vacuum of 100 milifar,
   at -80°C, for 72 hours using a Verdis Freeze Mobil 24.

   Assay for activity:
25   The assay was carried out in a 1 ml volume of 50 mM
   phosphate buffer pH 8.0, containing 10 μM pyridoxal phosphate
   and 10 mM methionine for 10 min. at 37°C with varying amounts
   of enzyme. The reaction was stopped by adding 0.5 ml of 4.5%
   TCA. The suspension was centrifuged at 15 K rpm for 2 min.
30 0.5 ml of supernatant with 0.5 ml of 0.05% 3-methyl-2-
   benzothiazolinone hydrazone in 1 ml of 1 M sodium acetate pH
   5.2 was incubated at 50°C for 30 min. And α-Ketobutyrate was
   then determined by spectrophotometry at OD_{335}. The amount of
   protein was determined by the procedure of Lowry Reagent Kit

(Sigma). The specific activity was calculated as units/mg
protein.

        The activity of rMETase were compared, and the results
showed no big difference between different formulations.

Example 15

Chemical Modification of Recombinant Methioninase

        The purified rMETase was formulated in a 0.15 M sodium
chloride in 10 mM sodium phosphate buffer (pH 7.2) at a
concentration between 0.1 M and 0.2 M. The activity was
approximately 20 units/mg.

        M-SC 5000 PEG molecular weight 5000 (Methoxy-SC-PEG, MW
5000 from Shearwater polymers Inc.), was dissolved in 20 mM
sodium phosphate buffer (pH 8.3) at a concentration between
2mM and 20 mM. The molar rations of M-SC 5000 PEG to rMETase
are varied from 10 : 1 to 120 : 1.

        The PEGylation reactions were carried out in reaction
buffer (25 mM sodium phosphate buffer, pH 8.3), at 20°C for 60
minutes. The reactions were stopped with stop buffer (0.14 M
sodium phosphate buffer, pH 6.5) at 0°C. Unreacted M-SC 5000
PEG was then removed with 30 K Amicon Centriprep
Concentrators. The resulting PEG - methioninase was
formulated in 0.15 M sodium chloride and 10 mM sodium
phosphate (pH 7.2) while centrifuging with 30k Amicon
Centriprep concentrators.

Analysis of PEG - rMETase *in vitro*

        PEG-rMETase were analyzed by activity assay,
electrophoresis and HPLC, Figures 14-16.

Activity Assay

        The activity of PEG-rMETase were between 80% to 20% of
the unmodified rMETase.

Electrophoresis

        PEG-rMETase were applied to both native and SDS-PAGE.

HPLC analysis:

5   PEG-rMETase were applied to a gel filtration column, no
original rMETase peak was detected, only the PEG - METase peak
were observed. The retention time (RT) were shorter along
with the molecular ratios of PEG and rMETase increased.

10 Pharmacokinetics of PEG-rMETase:
        Purified endotoxin-free PEG-rMETase were injected into
the tail-vein of mice. The blood samples were collected every
two hours. The levels of rMETase were measured by activity
assay (Figure 16).

15

Example 16
Efficacy and Toxicity of Recombinant Methioninase

Growth Inhibition of Cells by rMETase *in vitro*
20   KB3-1 cells (Human squamous cell carcinoma) were grown in
RPMI 1640 medium supplemented with 10% FBS. Various
concentrations of rMETase were added to the medium and
incubated at 37°C, 5% CO_2. The relative cell number was
measured at OD_{570}. The results demonstrated that rMETase
25 effectively inhibited cell growth (Figure 17).

Growth Inhibition of H460 and HT29 by rMETase *in vitro*
        Human lung cancer cells H460 and Human colon cancer cells
HT29 were grown with different concentrations of rMETase (0- 4
30 units/ml) for 4 days, then the live cells were counted. The
experiments were repeated three times. The results
demonstrated that rMETase effectively inhibited both H460 and
HT29 cell growth (Fig 25).

Comparisons of the sensitiveties to rMETase between
normal cells and Human cancer cells *in vitro*

Normal human foreskin fibroblasts HS-68 and human
keretinocytes were used as controls and compared with human
5   colon cancer SW-620 and human lung cancer cells H322m. The
cells were grown with different concentrations (0-4 units /ml)
of rMETase for three days and then the live cells were
counted. The experiments were repeated three times. The
results showed that both SW- 620 and H322m cells were dead at
10   the concentration 1 unit / ml of rMETase, the normal human HS-
68 cells were still alive although the growth rate was
reduced. The normal human keretinocytes were also much less
sensitive to rMETase compared to cancer cells (Fig 26).

15   Growth Inhibition of Cells by rMETase in Nude mice
2 X 10^5 cells were injected into Balb/c nu/nu, female,
mice in groups of eight. Control: normal saline. Group I:
rMETase 30 units, Group II: rMETase 100 units; ip twice a day
from day 5 to day 14. The tumor size and body weight were
20   measured. The blood was collected on day 18. The results
demonstrated that rMETase effectively inhibited tumor growth
without loss of body weight and without any effect on blood
cell production (Figures 18-22).

25   Growth Inhibition of H460 and HT29 Cells by rMETase in
nude mice.
10^6 H460 cells and HT29 cells were transplanted s.c.,
respectively, rMETase 40 units or 100 units twice a day were
given by ip injection from day 2 to day 16. The control group
30   had ip injection with normal saline 0.2 ml twice a day. The
mice were sacrificed at day 16. The results demonstrated that
rMETase effectively inhibited tumor growth without body weight
loss and without effect on blood cell production.

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Pilot Phase I Clinical Trial of purified, recombinant METase

Patient 1, female, 50 years old, with stage IV breast carcinoma with lymph nodes metastasis, received 10,000 units
5   (0.5g) rMETase iv infusion for 10 hours.

Patient-2, 48 years old, female, with state IV breast carcinoma with lymph nodes metastasis, received 5,000 units (0.25g) rMETase by in infusion for 24 hours.

Patient-3, 56 years old, female, with stage III renal
10   carcinoma, received 10,000 units (0.5g) rMETase by iv infusion for 24 hours. Table 14.

Physical examinations were recorded and the blood samples were collected before treatment, during treatment every two hours and two hours and up to 48 hours (as indicated) after
15   the end of the infusion. Laboratory determinations were carried out according to WHO criteria.

The results showed that the rMETase levels were enhanced immediately after the start of the infusion and maintained high level during the infusion in all patients. In one
20   patient, after 48 hours, the methioninase level dropped back base line. The results showed that the rMETase level was enhanced immediately after the start of the infusion, reached the highest point at 10 hours. Eight hours after the infusion was stopped the level was 50% of the peak and was still
25   maintained at 20% of the peak 16 hours after the end of the infusion. The results of the laboratory examinations were evaluated according to WHO criteria and showed no acute toxicity in patient-1, 2 or 3. Tables 15 and 16 and Figures 23 and 24. The result suggested that rMETase did not cause any
30   toxicity

Table 14			
Protocol of Clinical Phase I Trial for Recombinant Methioninase			
	Patient I	Patient II	Patient III
Diagnosis	Breast cancer with metastasis		Renal cancer
Sex	Female	Female	Female
Age	46	48	56
rMETase	10,00 units	5,000 units	10,000 units
I.v. infusion	8 hours	24 hours	24 hours
Blood collection	Before infusion and during infusion every two hours, after infusion 48 hours		
Evaluation	WHO criteria		

Table 15			
Toxicity of Clinical Phase I Trial for Recombinant Methioninase			
Physical & Laboratory	Grade		
Examination	Patient I	Patient II	Patient II
Hematological	0	0	0
Gastrointestinal	0	0	0
Renal	0	0	0
Pulmonary	0	0	0
Fever	0	0	0
Allergic	0	0	0
Phlebitis	0	0	0
Cutaneous	0	0	0
Cardiac	0	0	0
Neurological	0	0	0

Table 16					
Pilot Phase I Clinical Trial, record of vital statistics in Patient I					
(rMETase 10,000 units iv infusion for 8 hours)					
Time					
		Temp.	Pulse	respir. rate	Blood press.
Before infusion		36.70	61	20	114/76
During infusion	1h	36.60	58	18	116/78
	2h	36.60	59	20	118/80
	3h	36.60	62	20	112/74
	4h	36.70	64	20	112/74
	5h	36.50	62	20	116/76
	6h	36.60	64	20	116/78
	7h	36.60	64	20	114/78
	8h	36.60	62	20	114/76
After infusion	10h	36.60	62	20	114/76
	24h	36.60	60	18	114/76

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Claims

1.   A chemically modified methioninase comprising methioninase conjugated to a polymer.

2.   The chemically modified methioninase of claim 1 wherein the polymer is a polyalkylene oxide.

3.   The chemically modified methioninase of claim 2 wherein the polyalkylene oxide is polyethylene glycol.

4.   The chemically modified methioninase of claim 1 wherein the methioninase is substantially free of endotoxin.

5.   Isolated methioninase in crystalline form.

6.   Isolated methioninase in lyophilized form.

7.   Isolated methioninase having a specific activity of at least about 20 units/mg and being at least about 95% as determined by HPLC analysis.

8.   A therapeutic composition comprising a therapeutically effective amount of the methioninase of any one of claims 1-7.

9.   A method of treating a patient having a tumor comprising the step of administering to said patient a therapeutically effective amount of the methioninase composition of claim 8.

10.   A method of diagnosing a tumor in a patient comprising the steps of:
    a)   depleting ^{12}C methionine in the patient by administering methioninase to the patient;
    b)   repleting methionine in the patient by administering ^{11}C methionine to the patient; and

c)   detecting the presence of elevated levels of ^{11}C
methionine in the tumor cells of the patient.

11.   The method of claim 10 wherein said methioninase is
the composition of claim 8.

12.   A method of treating or preventing cardiovascular
disease in a patient comprising the step of administering to
said patient a therapeutically effective amount of methioninase
wherein the methioninase is substantially free of endotoxin.

13.   The method of claim 12 wherein said methioninase is
the composition of claim 8.

14.   The method of claim 12 wherein the cardiovascular
disease is arteriosclerosis.

15.   A method of lowering homocysteine levels in a patient
comprising the step of administering to said patient a
therapeutically effective amount of methioninase wherein the
methioninase is substantially free of endotoxin.

16.   The method of claim 15 wherein said methioninase is
the composition of claim 8.

17.   A high-level expression module encoding
methioninase, said module comprising a nucleotide sequence
encoding methioninase operably linked to a promoter sequence,
wherein said promoter sequence is capable of directing the
expression of methioninase in a host cell such that the
expression of methioninase is from about 5-75% of the total
protein produce by said host.

18.   The expression module of claim 17, wherein said
promoter is an RNA polymerase promoter.

19.   The expression module of claim 18, wherein said
promoter is the T7 RNA polymerase promoter.

20.   The expression module of claim 17 wherein said
5   methioninase-encoding nucleotide sequence is isolated from an
organism selected from the group consisting of *Pseudomonas
putida, Trichomonas vaginalis, Nippostrongylus brasiliensis*, and
Fusobacterium sp.

10   21.   The expression module of claim 20, wherein said
nucleotide sequence is provided in Figure 8, or a degenerate
form thereof.

22.   The expression module of claim 21, wherein said
15   degenerate form of the nucleic acid sequence of Figure 8
comprises the sequence provided in Figure 8 that has been
altered to contain one or more codons that are more frequently
used in *E. coli*.

20   23.   A host cell transformed with the expression module
of claim 17.

24.   The host of claim 23, wherein said host is *E. coli*.

25   25.   The host of claim 24, wherein said host is *E. coli*
BL21(DE3).

26.   A vector containing the expression module of claim
17.
30

27.   An isolated nucleic acid molecule comprising the
nucleotide sequence provided in Figure 8 and one or more
nucleic acid sequences which encode two or more histidine
residues at the C- or N-terminals of the methioninase.
35

28. A method of identifying a transformed host cell that
expresses high levels of methioninase, said method comprising
the steps of:
      culturing a transformed host cell under conditions in
5   which methioninase is expressed; and
      selecting transformed host cells that are pink in color.

29. The method of claim 28, wherein said host cell is *E.
coli*.
10

30. The method of claim 29, wherein said host is
cultured on a solid media.

31. The method of claim 28, wherein said selected host
15 produces from about 5 to 75% of total cellular protein as
methioninase.

32. An improved gene therapy method, wherein said
improvement comprises the use of the expression module of claim
20 17.

33. The method of claim 32, wherein the expression of said
expression module is controlled by a tumor specific promoter.

25   34. A method to purify methioninase, said method
comprising the steps of:
      a) culturing a host expressing methioninase under
condition in which methioninase is expressed;
      b) heating an extract of a transformed cell that contains
30 methioninase in aqueous buffers from about 40-60°C for about 1-10
min.;
      c) centrifugation of the heated extract from about 10k to
20k rpm in a GS-3 rotor (Sorval Du Pont) for about 15 min. to 1
hour;
35   d) ultrafiltration of the supernatant using a filter of
about 50K to 100K pore size;

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        e)   DEAE ion exchange chromatography in low ionic strength
(from about 10-50mM) KCl at about pH 7.0-7.6, and collecting
fractions containing methioninase eluted in an about 40-200mM
KCl gradient;

  5   f)   a second DEAE ion exchange chromatography in medium
ionic strength (100-200mM) KCl at about pH 8.0-8.6, and
collecting fractions containing methioninase eluted in a
phosphate buffered about 0.1 to .2M KCl;

        g)   contacting said fractions collected in step (e) with a
 10   chromatography medium capable of absorbing endotoxin; and

        h)   collecting the eluant containing methioninase.

        35.   The method of claim 34, wherein said collected
methioninase has an activity of at least 10 units per milligram
 15   protein and has from about 1-100ng of endotoxin per mg protein.

        36.   The method of claim 34, wherein an Acticlean® Etox
column is used in step (g).
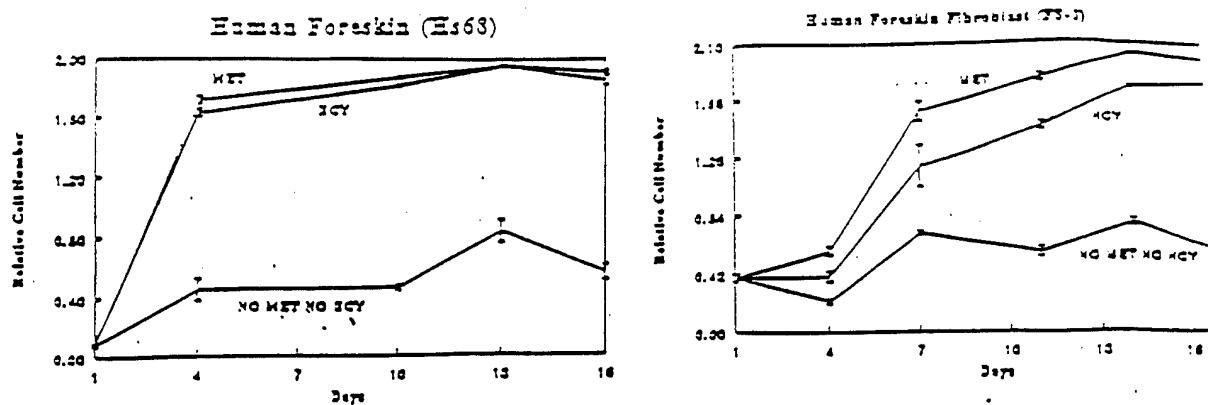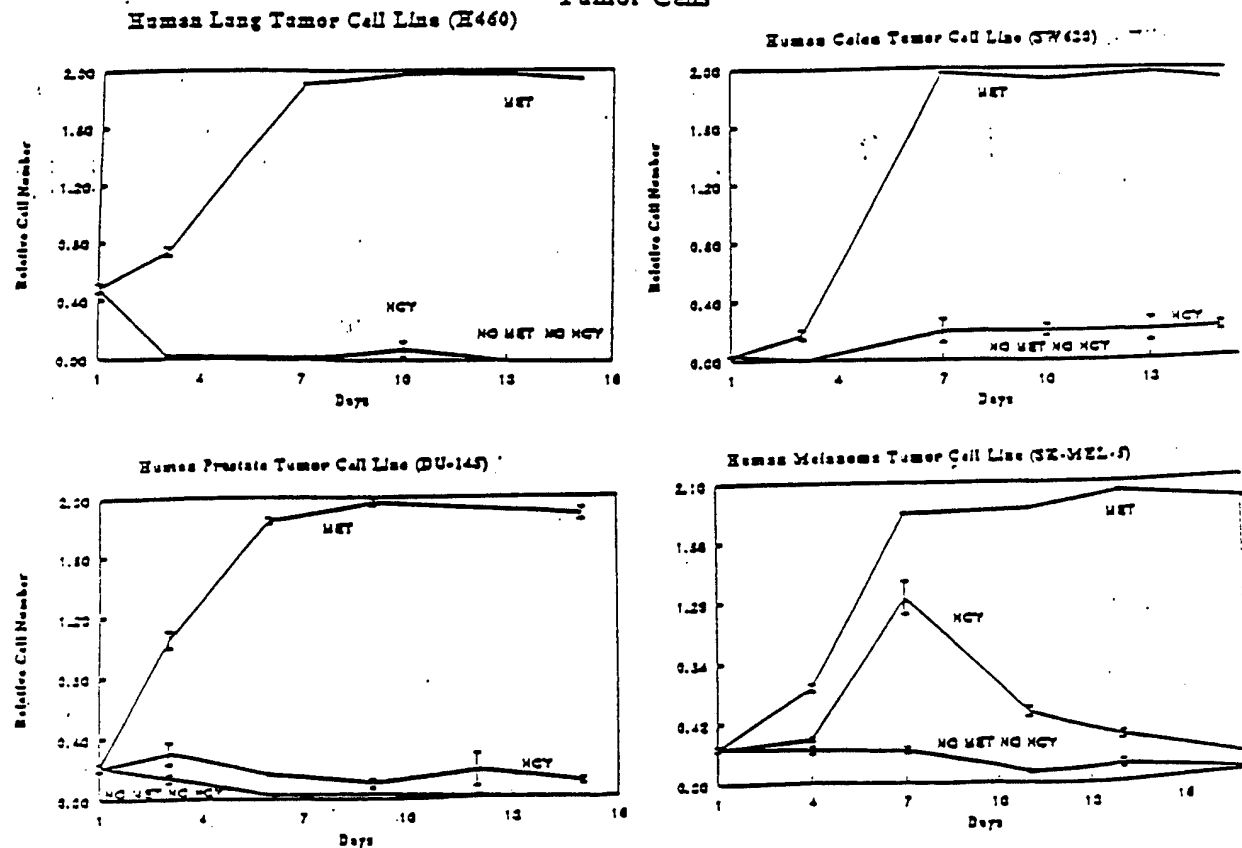
Normal Cells

Fig. 1
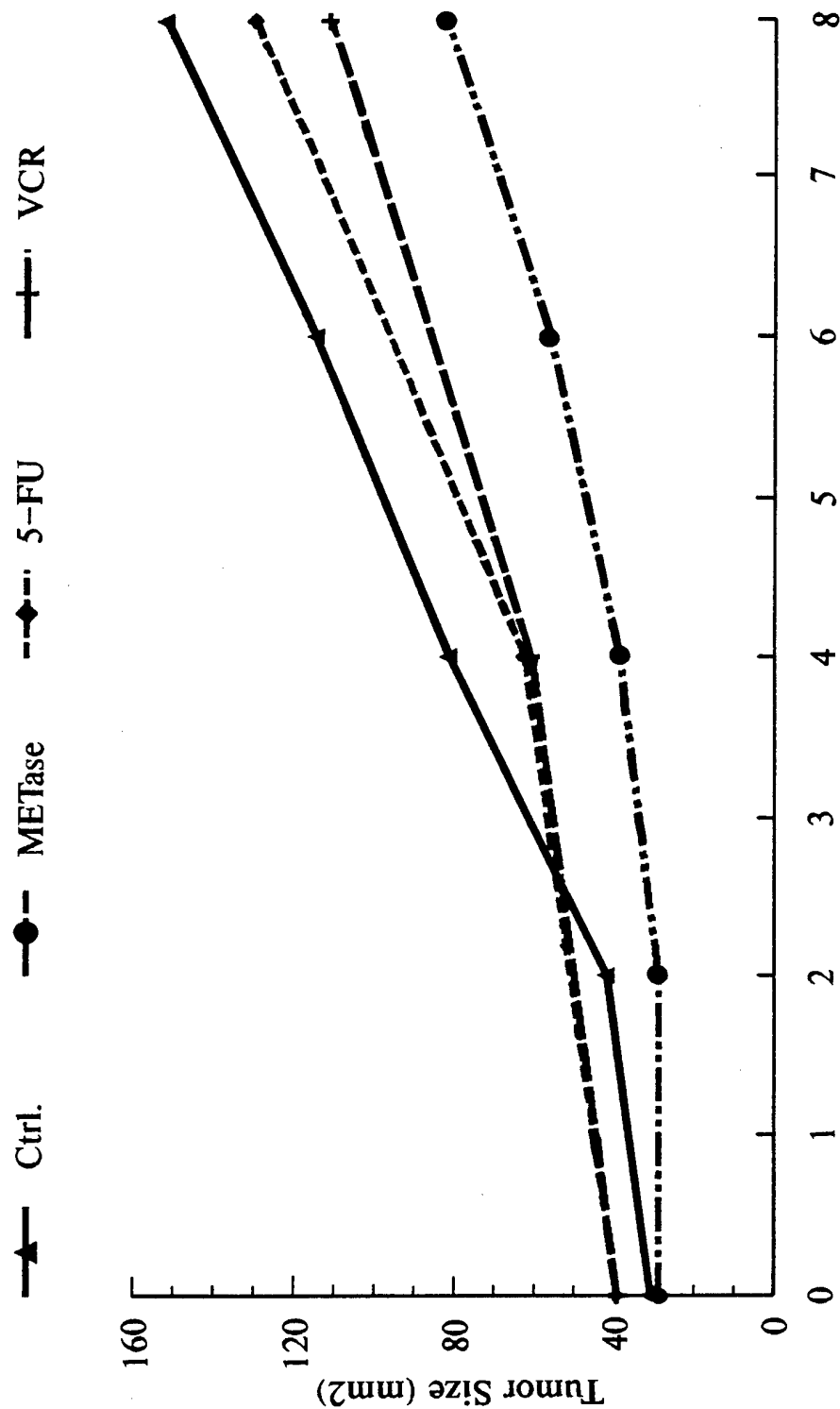
FIG. 2
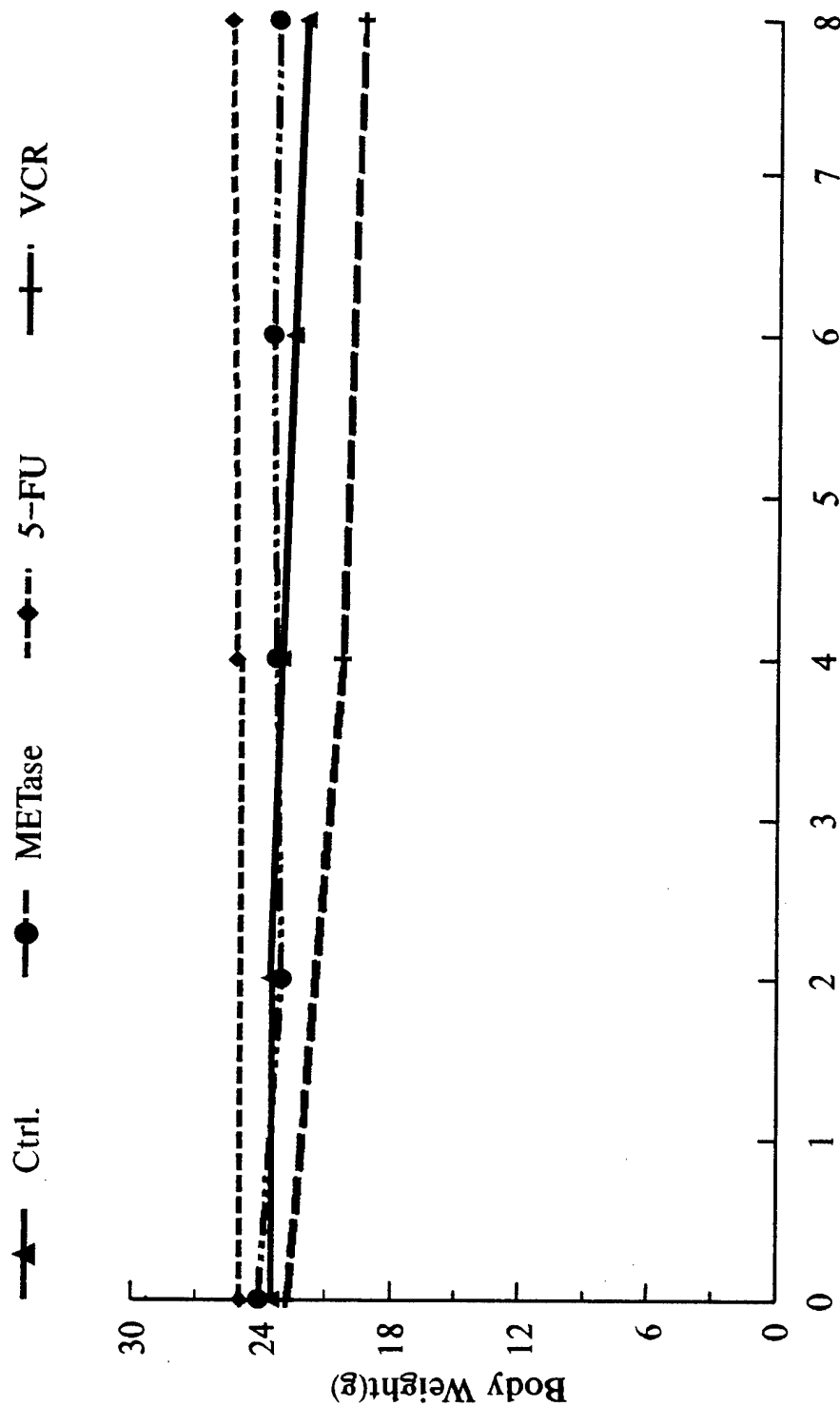
FIG. 3
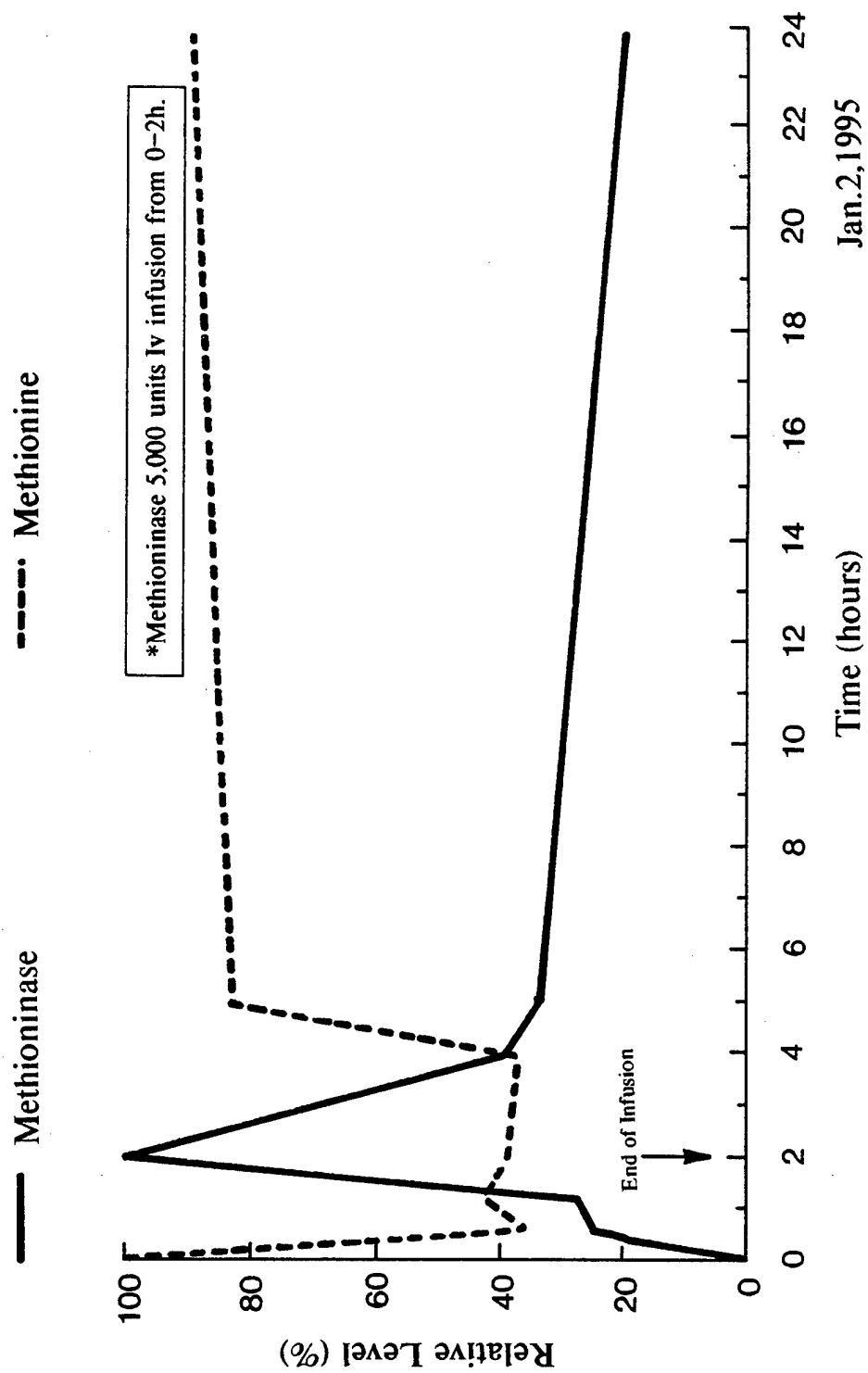
FIG. 4
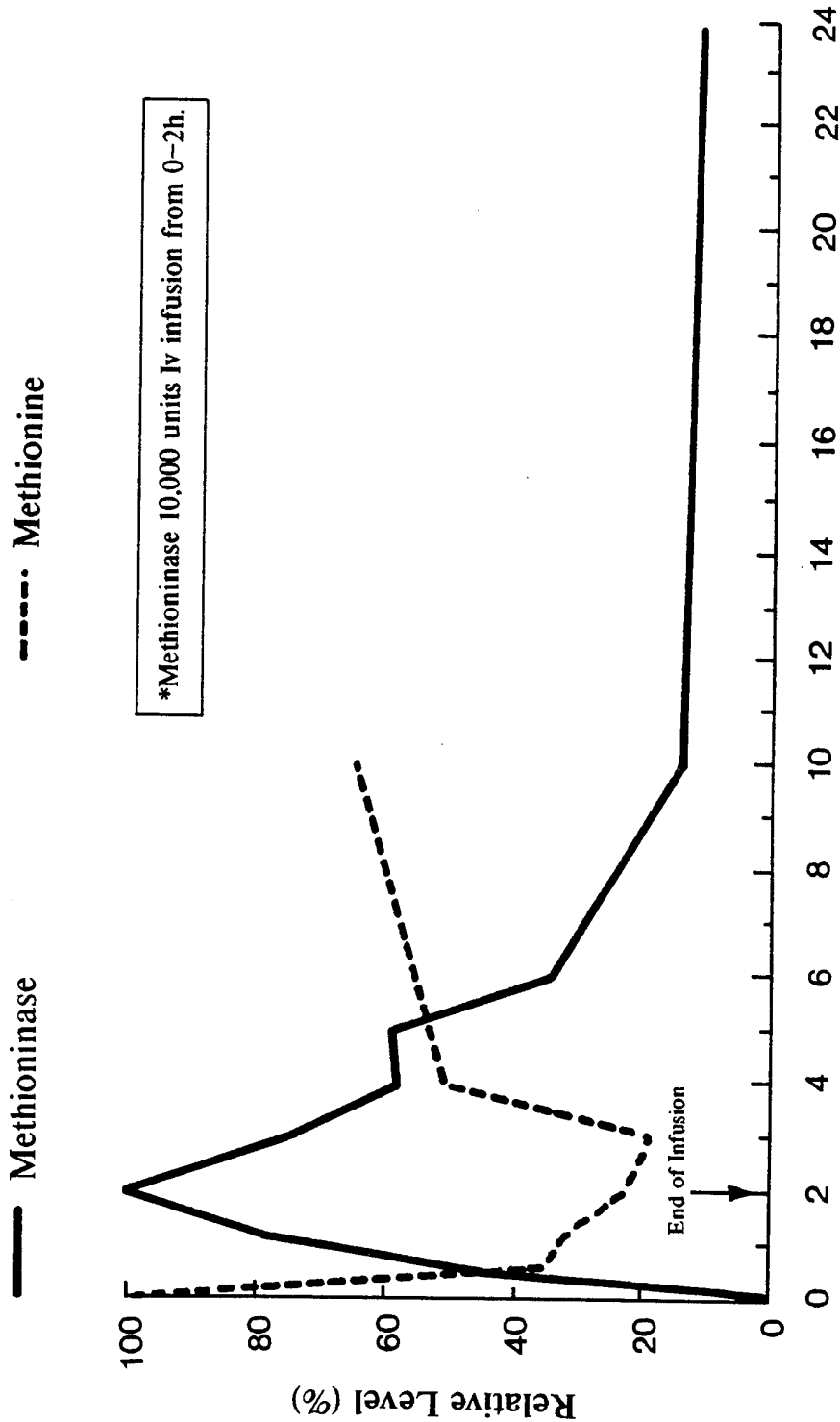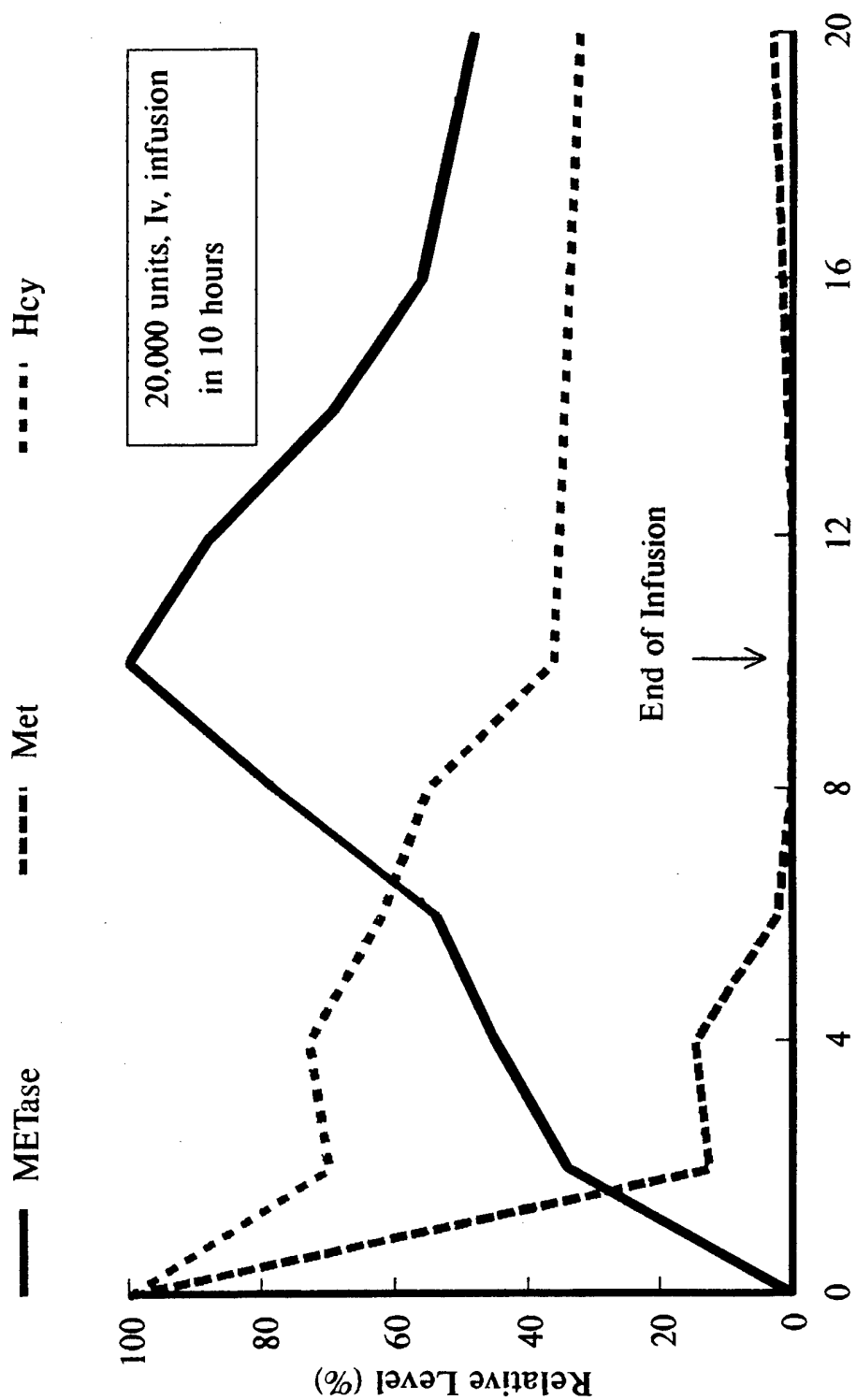
FIG. 5

FIG. 6
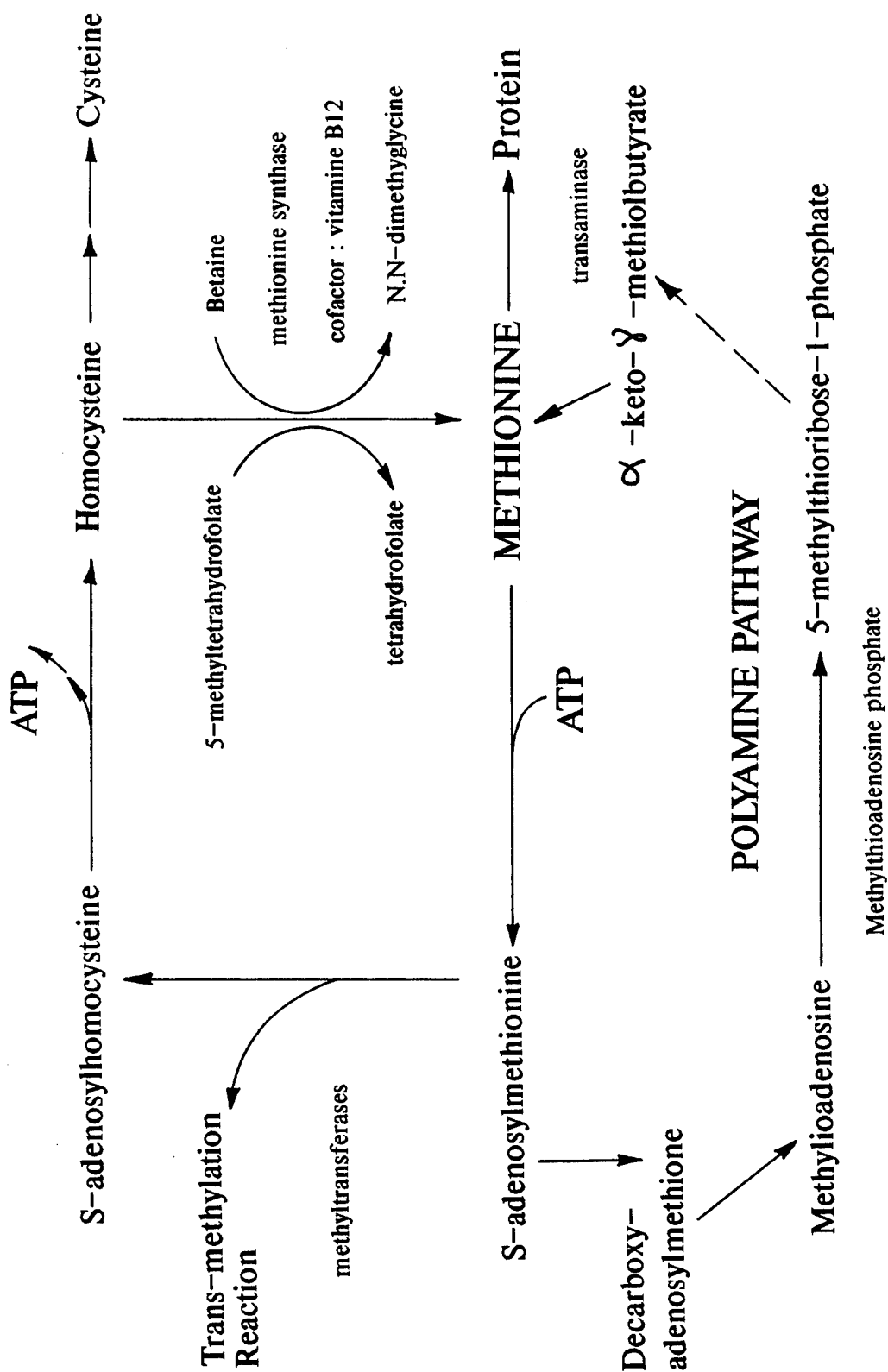
FIG. 7

```
GCCGGTCTGT GGAATAAGCT TATAACAAAC CACAAGAGGC GGTTGCCATG CACGGCTCCA    13
                                                        Met HisGlySer   4

ACAAGCTCCC AGGATTTGCC ACCCGCGCCA TTCACCATGG CTACGACCCC CAGGACCACG    73
AsnLysLeuPro GlyPheAla ThrArgAla IleHisHisGly TyrAspPro GlnAspHis     24

GCGGCGCACT GGTGCCACCG GTCTACCAGA CCGCGACGTT CACCTTCCCC ACCGTGGAAT   133
GlyGlyAlaLeu ValProPro ValTyrGln ThrAlaThrPhe ThrPhePro ThrValGlu    44

ACGGCGCTGC GTGCTTTGCC GGCGAGCAGG CCGGCCATTT CTACAGCCGC ATCTCCAACC   193
TyrGlyAlaAla CysPheAla GlyGluGln AlaGlyHisPhe TyrSerArg IleSerAsn     64

CCACCCTCAA CCTGCTGGAA GCACGCATGG CCTCGCTGGA AGGCGGCGAG GCCGGGCTGG   253
ProThrLeuAsn LeuLeuGlu AlaArgMet AlaSerLeuGlu GlyGlyGlu AlaGlyLeu    84

CGCTGGCCTC GGGCATGGGG GCGATCACGT CCACGCTATG GACACTGCTG CGCCCCGGTG   313
AlaLeuAlaSer GlyMetGly AlaIleThr SerThrLeuTrp ThrLeuLeu ArgProGly   104

ACGAGGTGCT GCTGGGCAAC ACCCTGTACG GCTGCACCTT TGCCTTCCTG CACCACGGCA   373
AspGluValLeu LeuGlyAsn ThrLeuTyr GlyCysThrPhe AlaPheLeu HisHisGly   124

TCGGCGAGTT CGGGGTCAAG CTGCGCCATG TGGACATGGC CGACCTGCAG GCACTGGAGG   433
IleGlyGluPhe GlyValLys LeuArgHis ValAspMetAla AspLeuGln AlaLeuGlu   144

CGGCCATGAC GCCGGCCACC CGGGTGATCT ATTTCGAGTC GCCGGCCAAC CCCAACATGC   493
AlaAlaMetThr ProAlaThr TrpValIle TyrPheGluSer ProAlaAsn ProAsnMet   164

ACATGGCCGA TATCGCCGGC GTGGCGAAGA TTGCACGCAA GCACGGCGCG ACCGTGGTGG   553
HisMetAlaAsp IleAlaGly ValAlaLys IleAlaArgLys HisGlyAla ThrValVal   184

TCGACAACAC CTACTGCACG CCGTACCTGC AACGGCCACT GGAGCTGGGC GCCGACCTGG   613
ValAspAsnThr TyrCysThr ProTyrLeu GlnTrpProLeu GluLeuGly AlaAspLeu   204

TGGTGCATTC GGCCACCAAG TACCTGAGCG GCCATGGCGA CATCACTGCT GGCATTGTGG   673
ValValHisSer AlaThrLys TyrLeuSer GlyHisGlyAsp IleThrAla GlyIleVal   224

TGGGCAGCCA GGCACTGGTG GACCGTATAC GTCTGCAGGG CCTCAAGGAC ATGACCGGTG   733
ValGlySerGln AlaLeuVal AspArgIle ArgLeuGlnGly LeuLysAsp MetThrGly   244

CGGTGCTCTC GCCCCATGAC GCCGCACTGT TGATGCGCGG CATCAAGACC CTCAACCTGC   793
AlaValLeuSer ProHisAsp AlaAlaLeu LeuMetArgGly IleLysThr LeuAsnLeu   264

GCATGGACCG CCACTGCGCC AACGCTCAGG TGCTGGCCGA GTTCCTCGCC CGGCAGCCGC   853
ArgMetAspArg HisCysAla AsnAlaGln ValLeuAlaGlu PheLeuAla TrpGlnPro   284

AGGTGGAGCT GATCCATTAC CCGGGCCTGG CGAGCTTCCC GCAGTACACC CTGGCCCGCC   913
GlnValGluLeu IleHisTyr ProGlyLeu AlaSerPhePro GlnTyrThr LeuAlaArg   304

AGCAGATGAG CCAGCCGGGC GGCATGATCG CCTTCGAACT CAAGGGCGGC ATCGGTGCCG   973
GlnGlnMetSer GlnProGly GlyMetIle AlaPheGluLeu LysGlyGly IleGlyAla   324

GGCGGCGGTT CATGAACGCC CTGCAACTGT TCAGCCGCGC GGTGAGCCTG GGCGATGCCG  1033
GlyTrpTrpPhe MetAsnAla LeuGlnLeu PheSerArgAla ValSerLeu GlyAspAla   344

AGTCGCTGGC GCAGCACCCG GCAAGCATGA CTCATTCCAG CTATACCCCA GAGGAGCGTG  1093
GluSerLeuAla GlnHisPro AlaSerMet ThrHisSerSer TyrThrPro GluGluArg   364

CGCATTACGG CATCTCCGAG GGGCTGGTGC GGTTGTCGGT GGGGCTGGAA GACATCGACG  1153
AlaHisTyrGly IleSerGlu GlyLeuVal TrpLeuSerVal GlyLeuGlu AspIleAsp   384

ACCTGCTGGC CGATGTGCAA CAGGCACTCA AGGCGAGTGC CTGAACCCGT CACGGATGAG  1213
AspLeuLeuAla AspValGln GlnAlaLeu LysAlaSerAla                        398
```

FIG. 8

FIG. 9

Expression Clone of rMETase

↓

Fermenatation   **Fermentation**

↓

Harvest & Disruption

↓

Heat & Ultrafiltration   **Purification**

↓

DEAE Sepharose FF (1)

↓

DEAE Sepharose FF (2)

↓

Acticlean

↓

Concentration & Sterilization   **Formulation**

↓

Pure rMETase
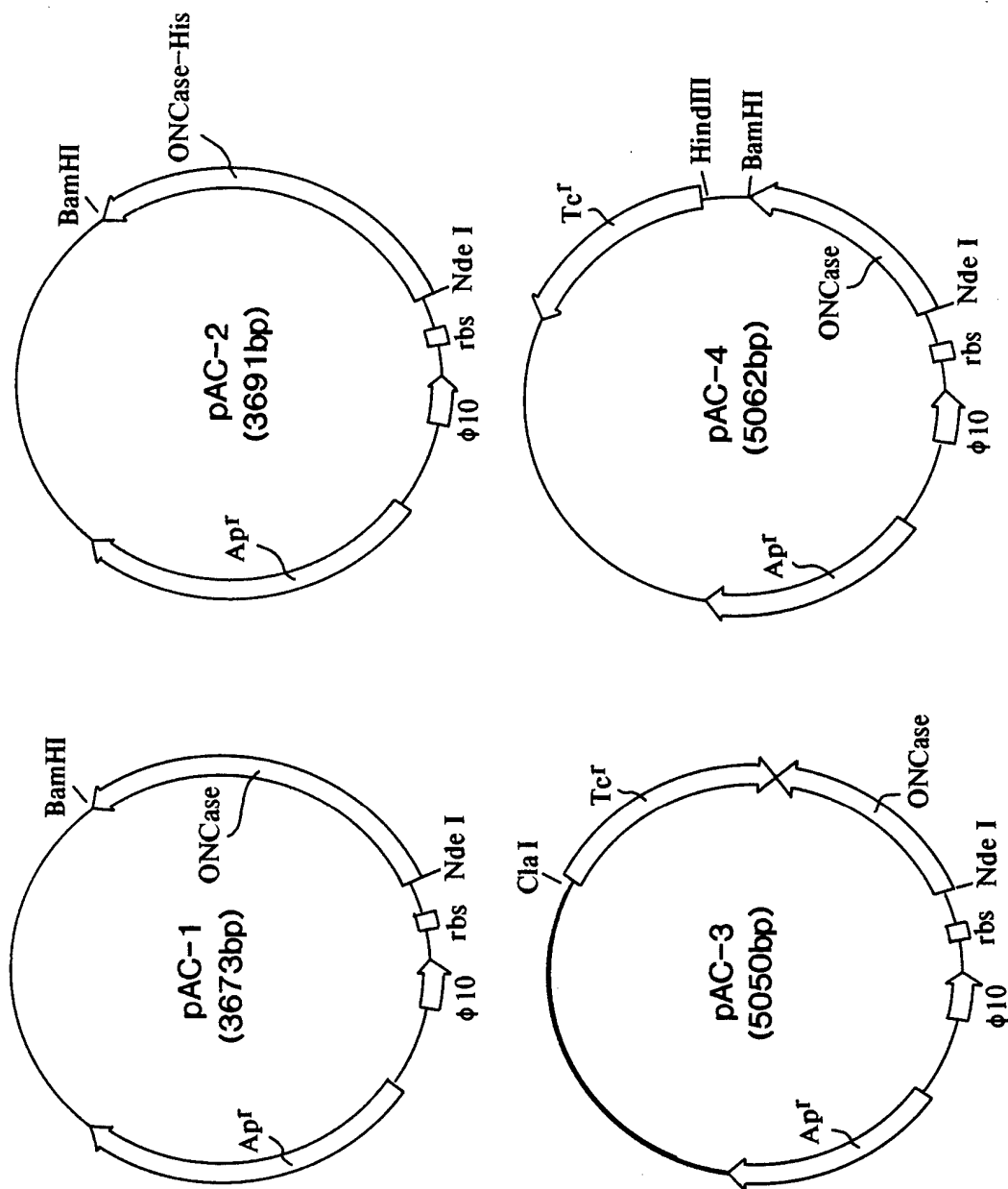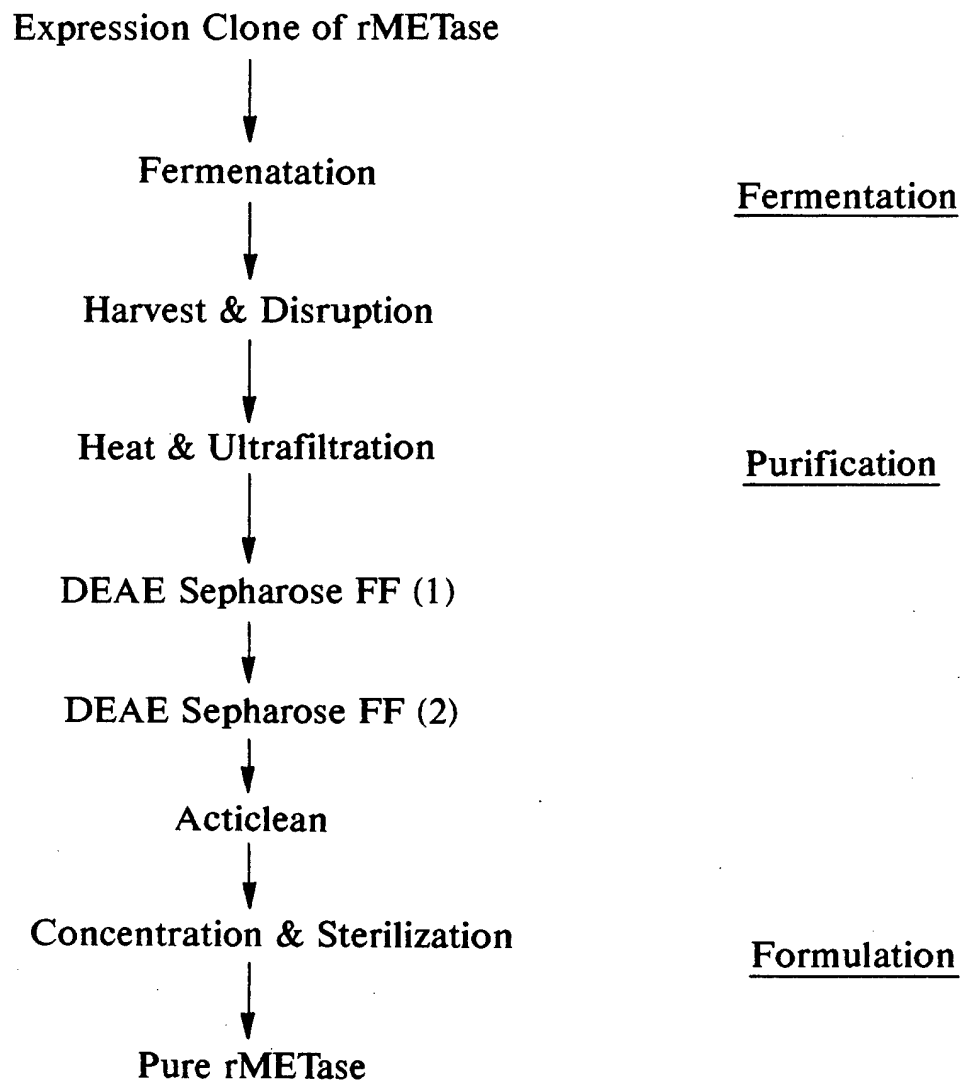
FIG. 10

Procedure (Batch 11)	Volume (ml)	Activity (units)	Protein (g)	S A * (units/mg)	Recovery Rate (%)	Yield (%)
Cell Lysis	5,000	82,000	29.3	2.8	100	100
Heat & UF**	4,500	77,000	19.3	4.0	94	94
DEAE-FF.1	2,200	65,400	6.0	10.9	85	80
DEAE-FF.2	800	57,500	2.88	20.0	88	70
Acticlean	182	52,000	2.59	20.1	90	63
Endotoxin			2 EU / mg			

April 2, 1996

FIG. 11

* S.A. : Specific Activity.　　** UF : Ultrafiltration.

SDS-PAGE

43kd ——►

1. 2.   3.

FIG. 12A

HPLC
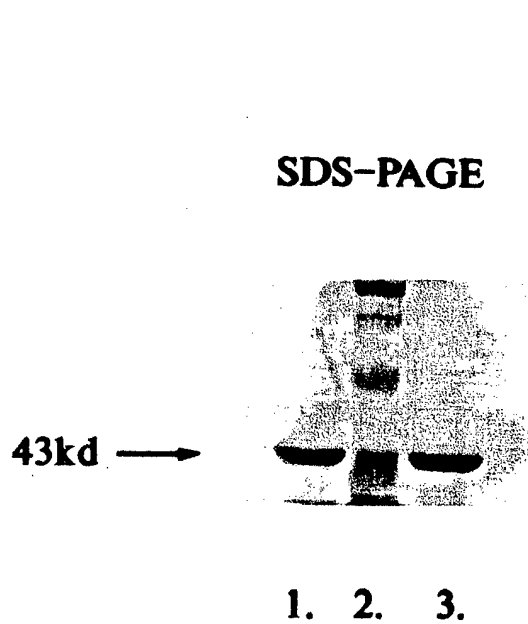
99% Purity

FIG. 12B
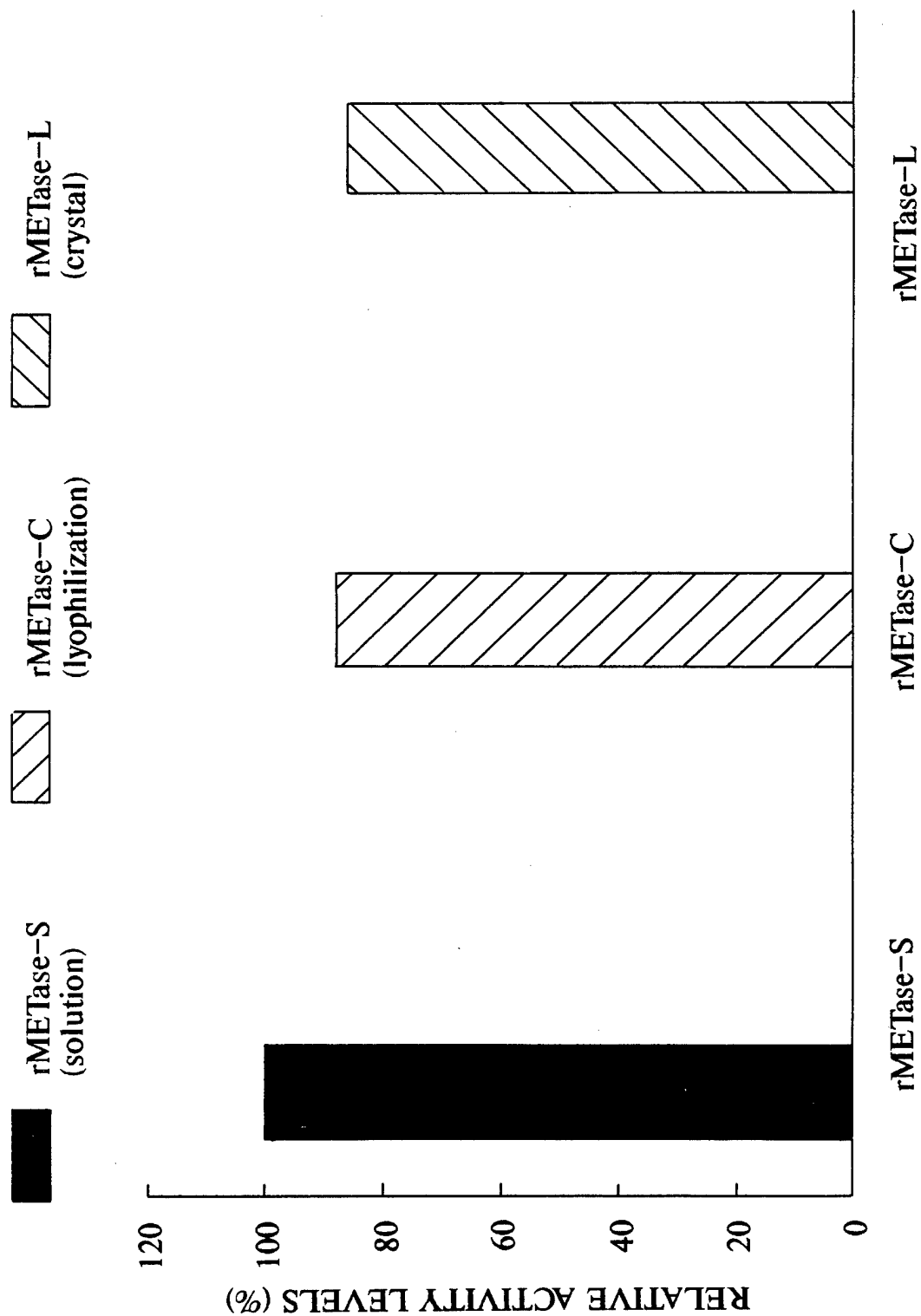
FIG. 13
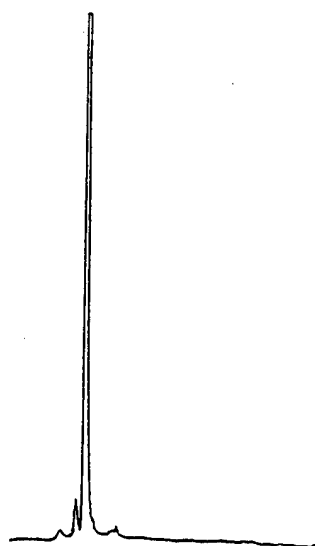
rMETase

FIG. 14A
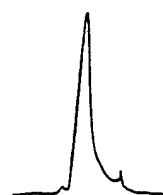
PEG-rMETase-1

FIG. 14B

PEG-rMETase-2

FIG. 14C
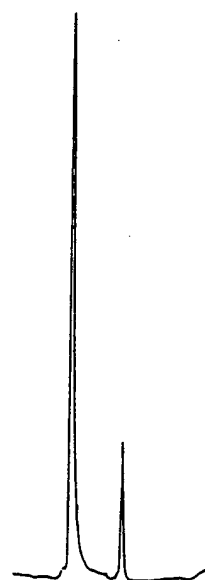
PEG-rMETase-3

FIG. 14D

FIG. 15
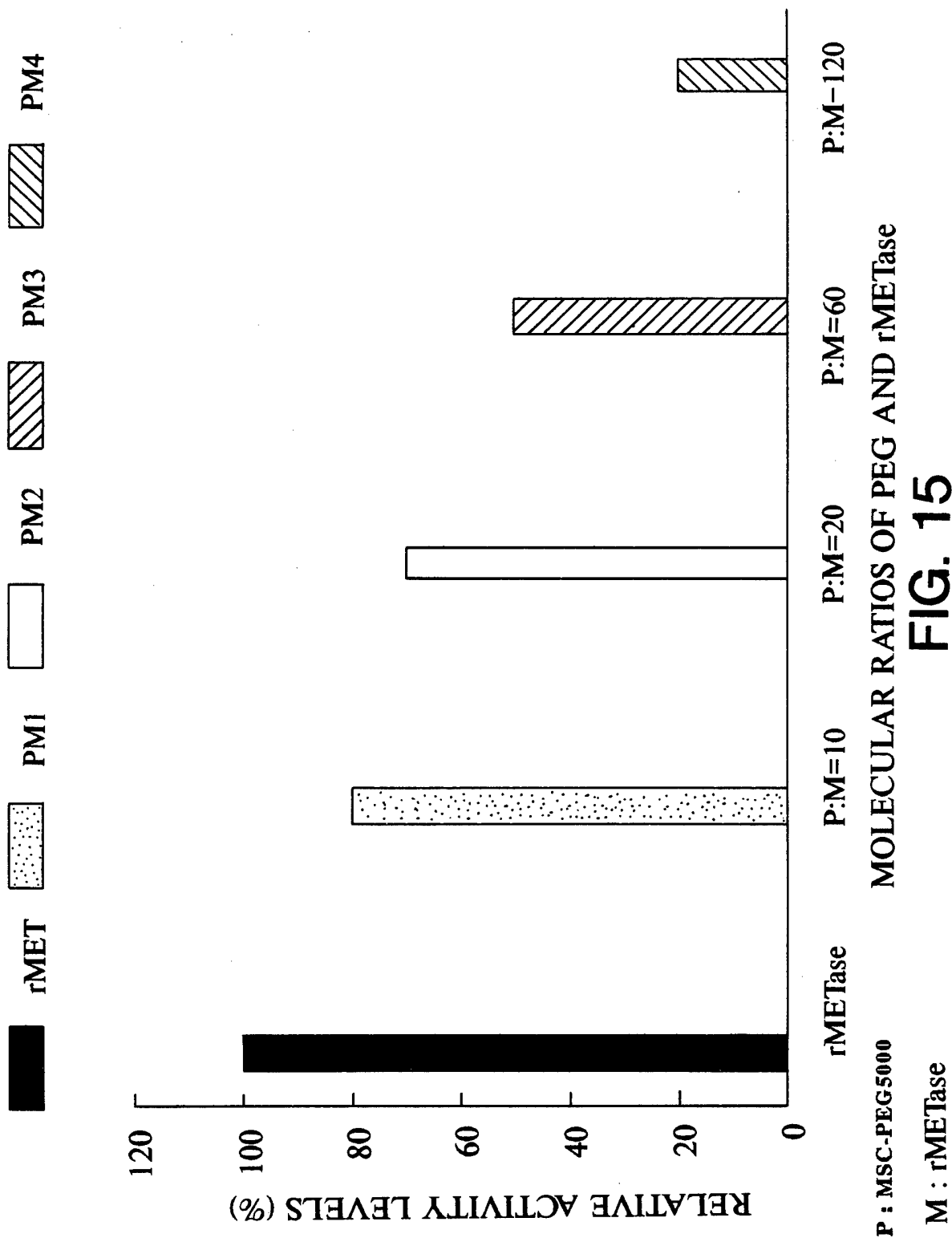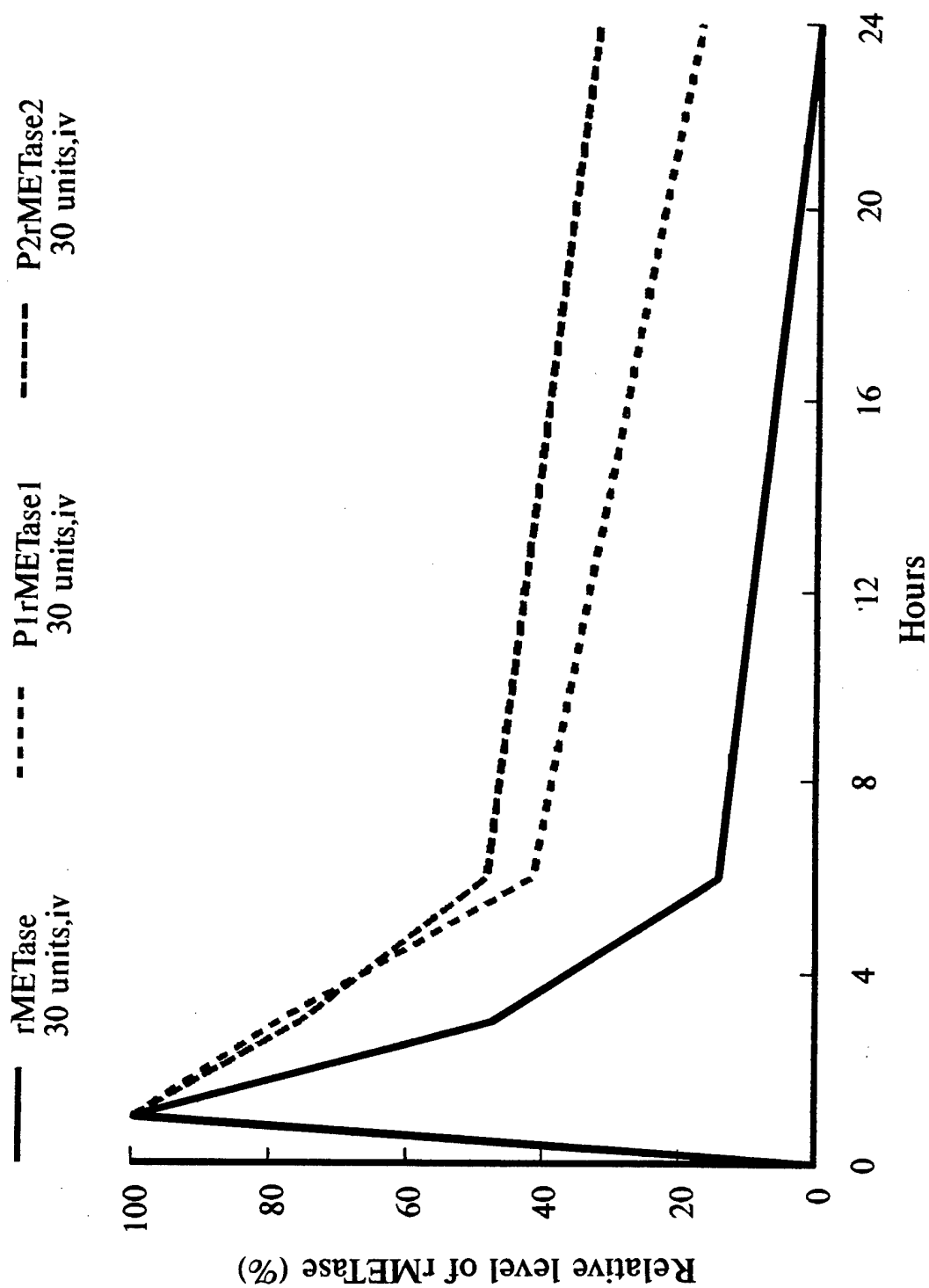
FIG. 16
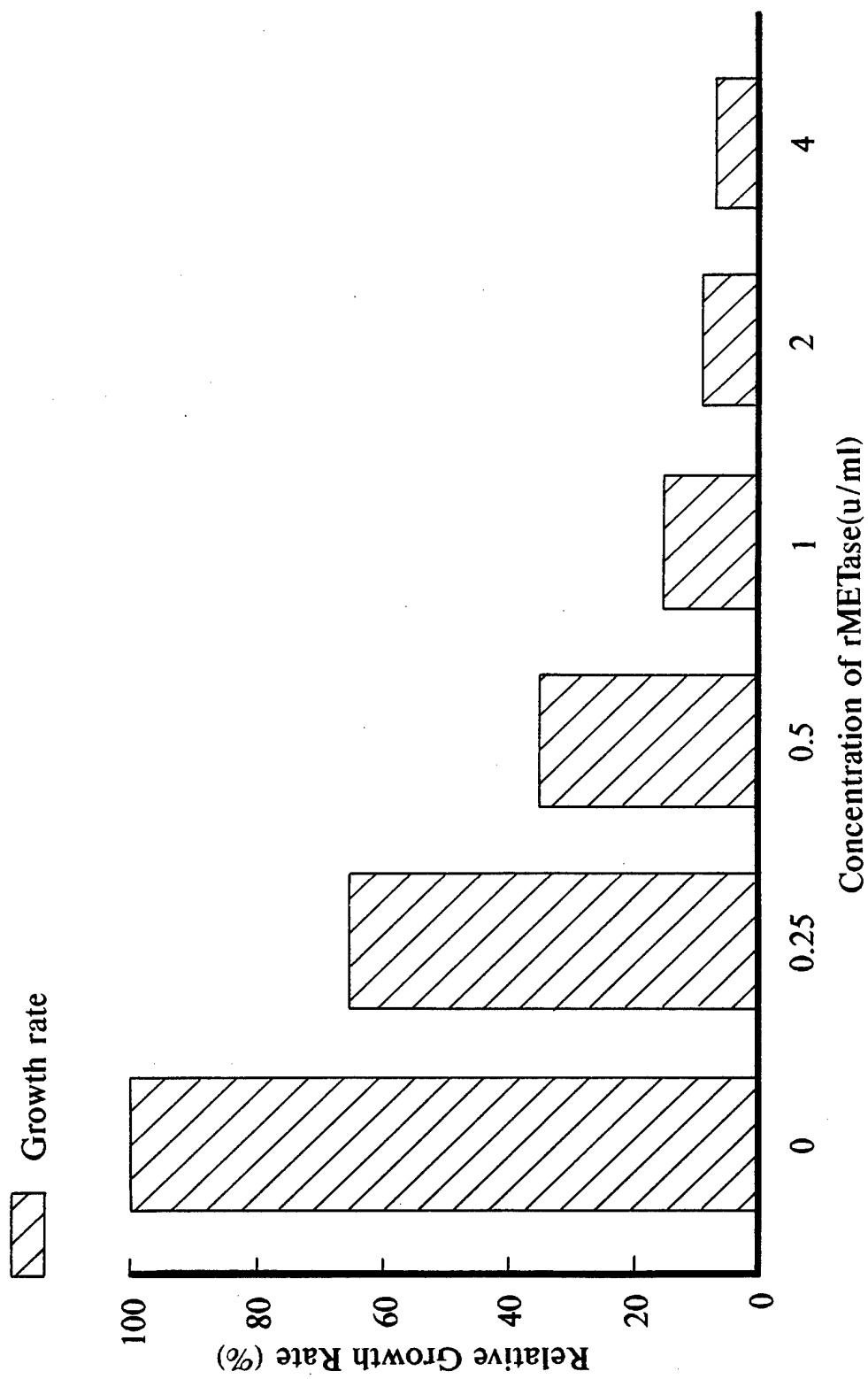
FIG. 17
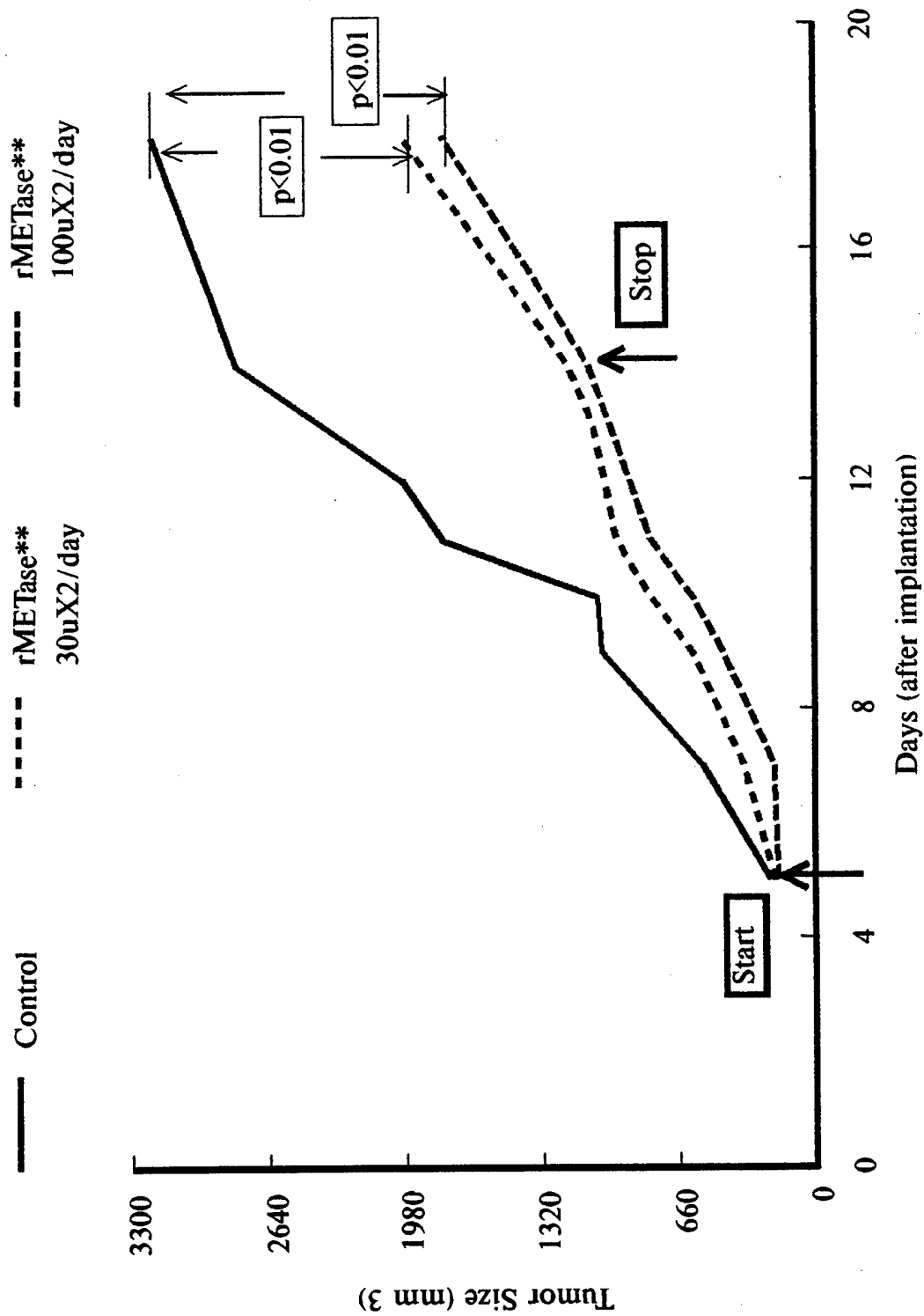
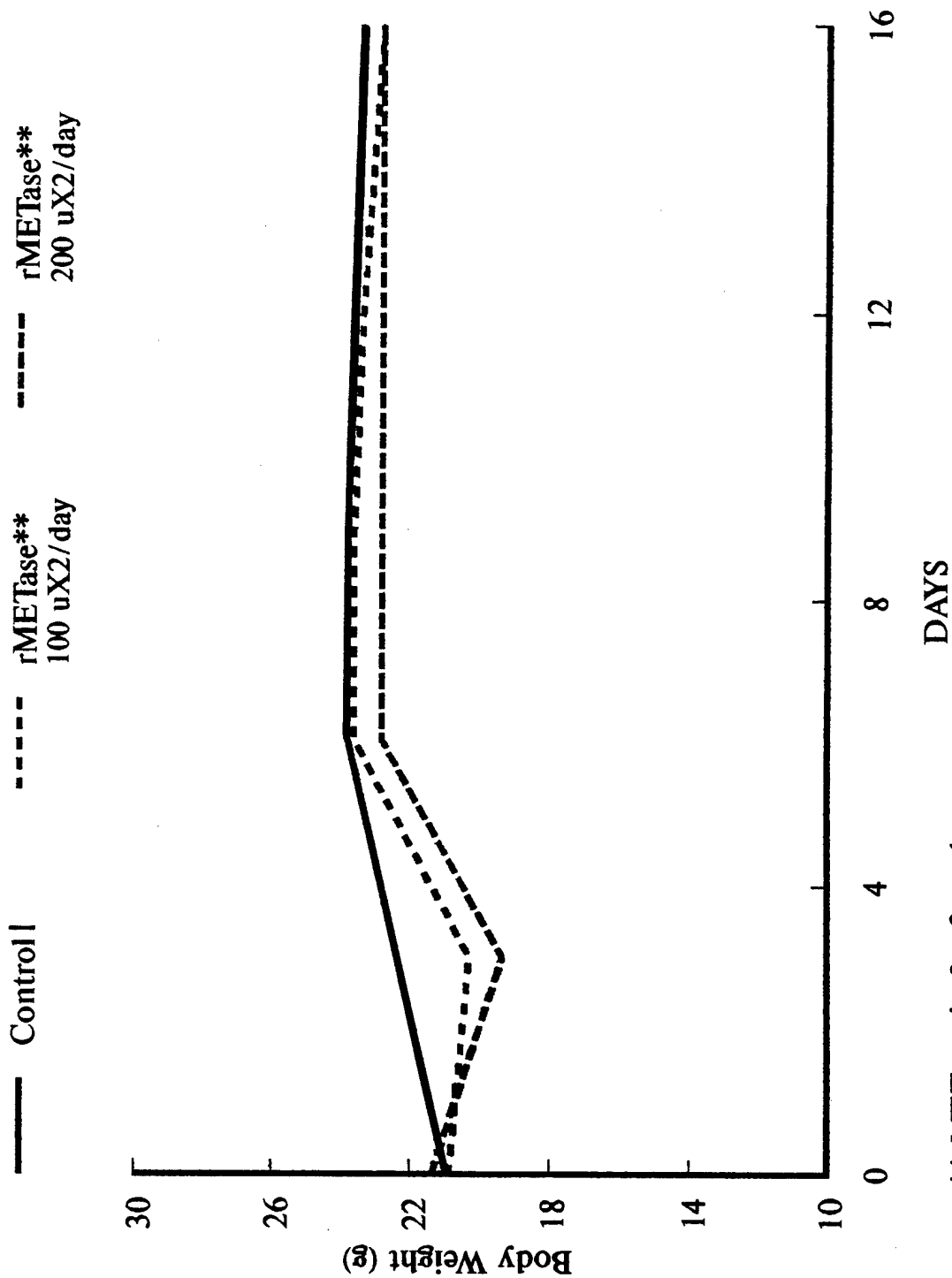FIG. 18

FIG. 19

*Human squamous carcinoma
**rMETase,ip,from day5 to day14
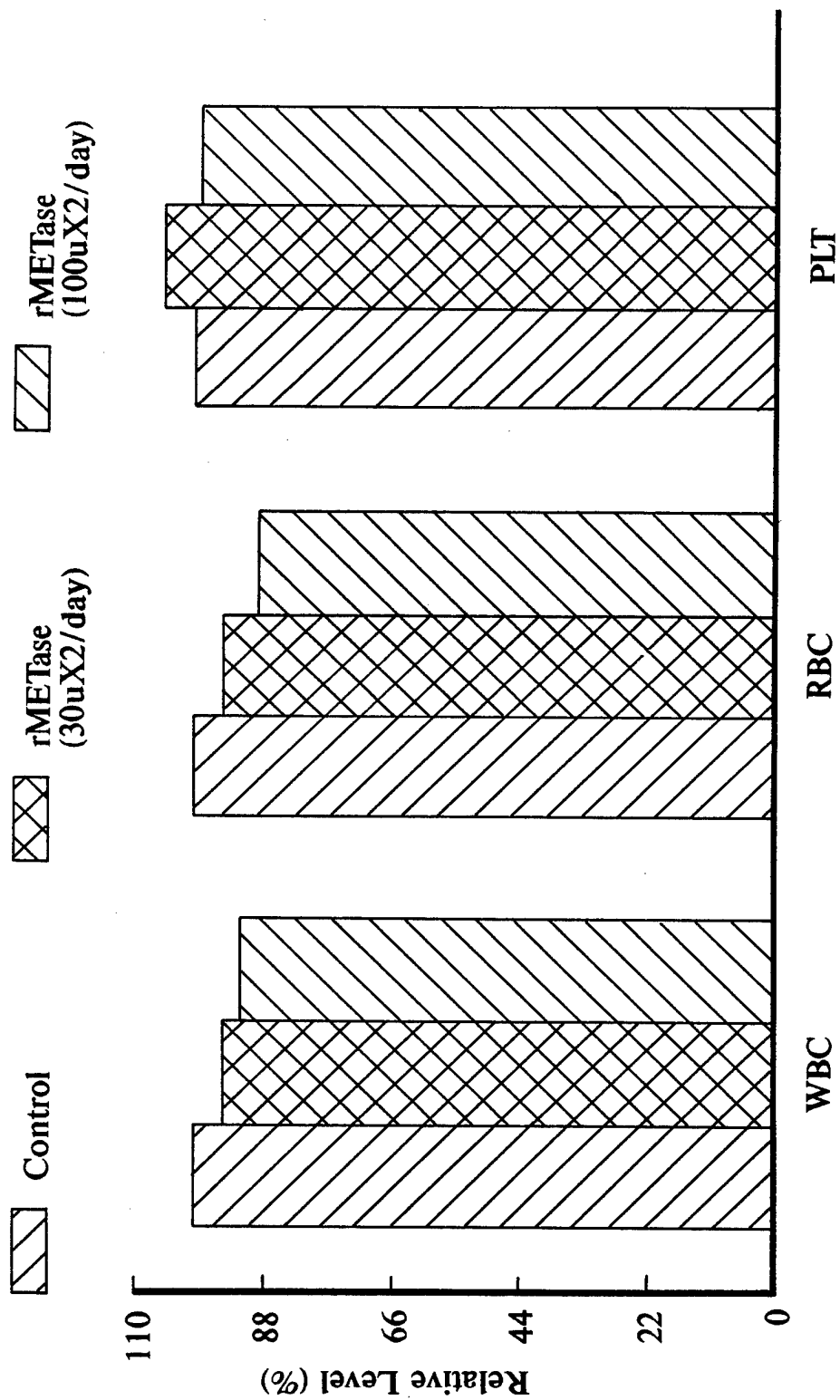
FIG. 20

*Human squamous carcinoma

FIG. 21

FIG. 22

BLOOD CELLS

**rMETase ip for 2 weeks

(CLINICAL PHASE I TRIAL)

Physical&Laboratory Examination	Grade		
	Patient 1	Patient 2	Patient 3
Hematological	0	0	0
Gastrointestinal	0	0	0
Renal	0	0	0
Pulmonary	0	0	0
Fever	0	0	0
Allergic	0	0	0
Phlebitis	0	0	0
Cutaneous	0	0	0
Cardiac	0	0	0
Neurological	0	0	0

* According to WHO toxicity criteria

FIG. 23
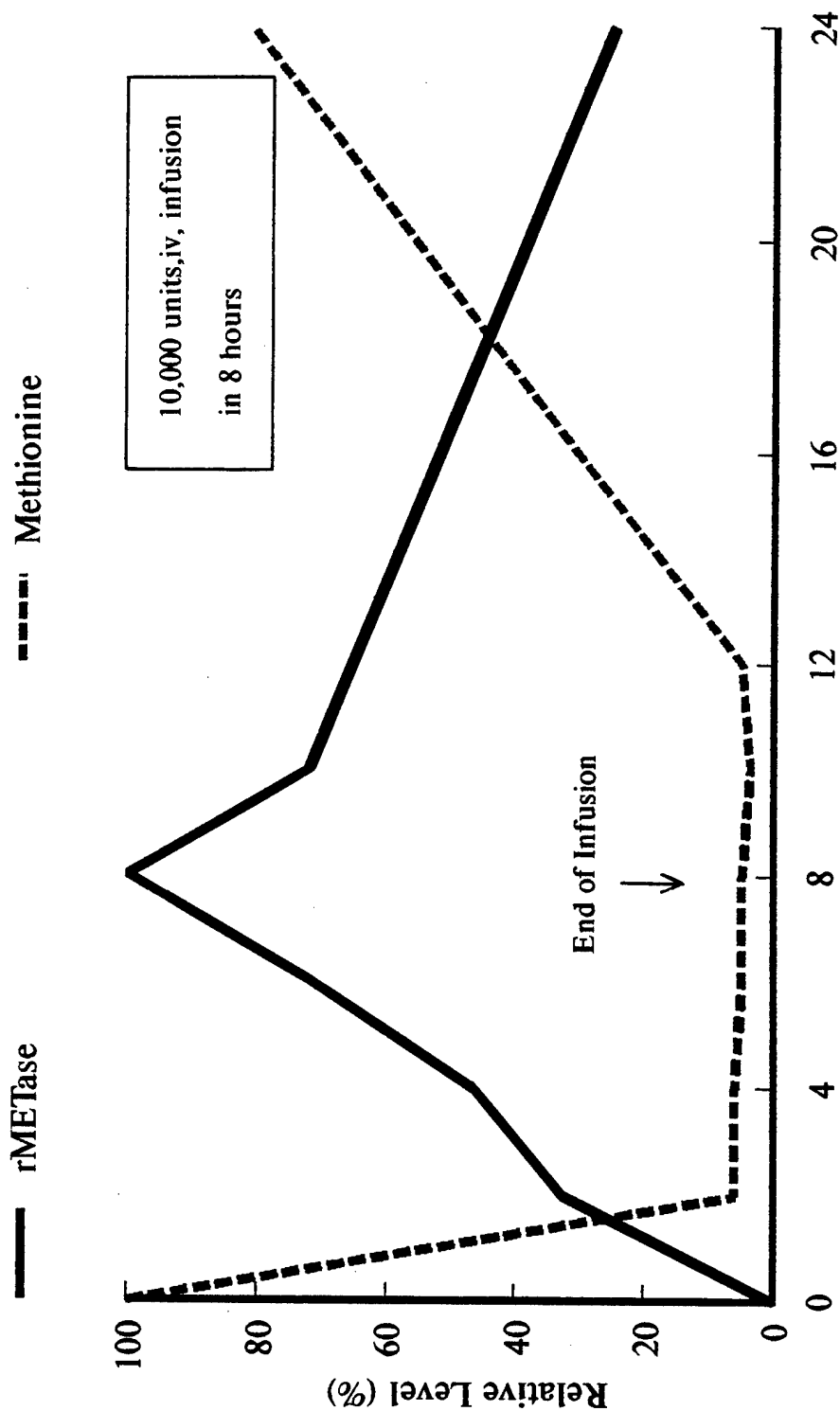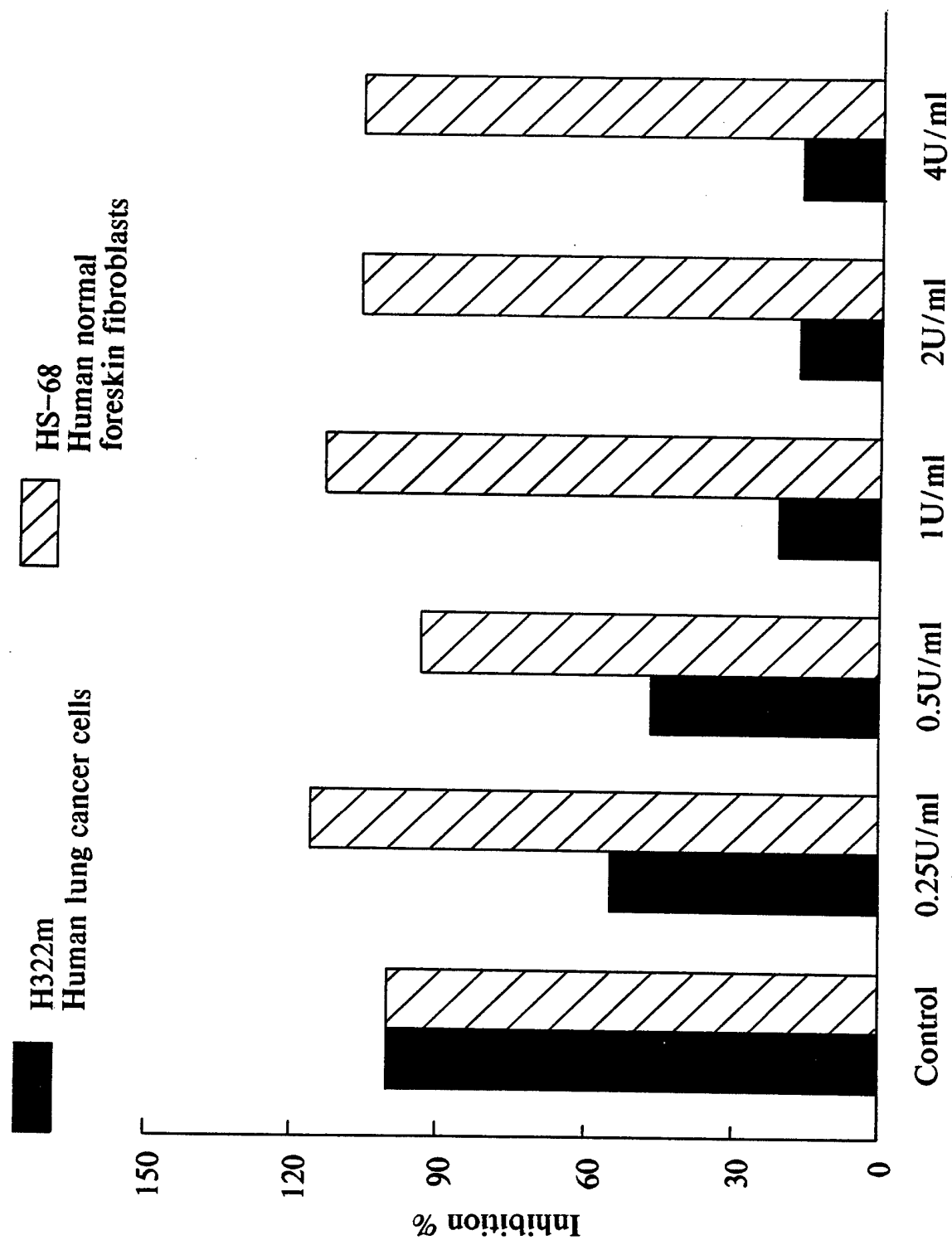
FIG. 24

FIG. 25

FIG. 26

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
IPC(6) :A61K 49/00; C12N 5/10
US CL :128/653.1, 898; 424/130.1; 435/40, 69.1; 562/559
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED
Minimum documentation searched (classification system followed by classification symbols)
U.S. : 128/653.1, 898; 424/85.1, 130.1; 435/40, 69.1, 252.3; 562/559

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS

Search Terms: e:methioninase, tumor, cancer, polymer...

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US, A, 5,028,420 (MASEGI ET AL.) 02 July 1991, see entire document.	1-36
Y	US, A, 5,487,984 (ALLET ET AL.) 30 January 1996, see entire document.	1-36
Y	US, A, 4,298,590 (BOGOCH) 03 November 1981, see entire document.	1-36
Y	JOURNAL OF THE NATIONAL CANCER INSTITUTE, Volume 82, No. 20, October 17, 1990, pages 1593-1660, (F. BREILLOUT ET AL.), Methionine Dependency of Malignant Tumors: A Possible Approach for Therapy	1-36

☐ Further documents are listed in the continuation of Box C.   ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance		
"E"	earlier document published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
12 SEPTEMBER 1996	0 2 OCT 1996
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer SAMUEL GILBERT
Facsimile No. (703) 305-3590	Telephone No. 703-308-3553

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